THE COMBINATIVE EFFECT OF SOLUBLE suLe^{A} GLYCAN AND SPECIALIZED BOVINE SEMEN EXTENDER, FSRD4+, ON BULL SPERM QUALITY

BY

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THESIS

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ABSTRACT

Artificial insemination is an invaluable tool that has been adopted by the cattle industry, primarily dairy, and continues to be a crucial reproductive technique. Likewise, cryopreservation is ubiquitously used in the U.S. as the preferred method to store bovine sperm due to convenience despite damage to sperm during processing and thawing. For this reason, one of our objectives was to determine if a liquid extender, FSRD4+, could extend the lifespan of sperm by days. Since longevity with minimal sperm damage was an ultimate goal, we also tested the effect on longevity by adding oviduct glycans to medium and extender. Before fertilization and while bound to the oviduct, sperm become quiescent and have prolonged viability and maintained motility. Additionally, sperm undergo capacitation to reach final maturation. Capacitation will allow the sperm to hyperactivate and penetrate the cumulus cells and the zona pellucida, and fertilize the oocyte. The sperm-oviduct binding and subsequent sperm reservoir formation is facilitated by glycan-lectin binding. In cattle, the Lewis A trisaccharide (Le^A) in the oviduct binds sperm. We tested sulfated Le^A, because it binds sperm with higher affinity, for its effects on sperm viability and motility compared to controls. Four variables were applied: temperature (5°C, 15°C, 22°C, 37°C), glycans ($suLe^A$, $suLe^X$, no glycan), medium (FSRD4+ and dmTALPC), and time (1, 24, 48, 72, 96, and 120 hr). Semen was collected and shipped overnight for experiments. Sperm were washed, incubated in their respective temperature, glycan, and media treatments, and analyzed for viability and motility. Viability was determined using fluorescent probes SYBR14 and propidium iodide (PI). Motility was determined as total motility where progressive and non-progressive motility were summed. Significance was determined by a p-value of ≤ 0.05. The results indicated viability had improved significantly in FSRD4+ extender had significantly higher viability than dmTALPC, both $suLe^A$ and $suLe^X$ performed similarly but

had significantly higher viability than no-glycan treatment, and 15˚C and 22˚C had significantly higher viability than 5˚C and 37˚C. Similarly, sperm motility was significantly higher in FSRD4+ extender compared to dmTALPC. Sperm motility was higher with $suLe^X$ compared to $suLe^A$ and no-glycan, and $suLe^A$ compared to no-glycan. Over the 120 hr, 15°C storage yielded significantly more viable, motile sperm than 5˚C, 22°C, and 37˚C, despite it having significantly lower motility than 22°C and 37˚C at the 1 hr time point. To summarize, sperm in FSRD4+ extender, with $suLe^A$, and at 15°C or 22°C had significantly higher viability, and sperm in FSRD4+ extender, with $suLe^X$ or $suLe^A$, and at 15˚C had significantly higher motility compared to other treatments. In conclusion, FSRD4+ and soluble glycans significantly improved sperm viability and motility during long-term liquid storage with the best combination of medium, glycan, and temperature as FSRD4+ extender, $suLe^A$, and 15˚C.

DEDICATION

I want to dedicate my thesis to my family for supporting me, and especially to my boyfriend, Adrian Guereca, for pushing me to finish my thesis. I can't wait to continue our lives together hopefully in Dallas.

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LIST OF ABBREVIATIONS

AI: artificial insemination

ART: assisted reproductive technologies

bi-SiaLN: biantennary 6-sialylated N-acetyllactosamine

BSA: bovine serum albumin

BSP1/2: binder of sperm proteins 1/2

CASA: computer assisted semen analysis

FITC-PSA: fluorescein isothiocyanate-labelled Pisum sativum agglutinin

ICSI: intracytoplasmic sperm injection

IVF: *in vitro* fertilization

(su)Le^{A}: (sulfated) Lewis^{A} trisaccharide

(su)Le^{X}: (sulfated) Lewis^{X} trisaccharide

NRR: non-return rate

PI: propidium iodide

ROS: reactive oxygen species

SCSA: sperm chromatin structure assay

TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling assay

UTJ: utero-tubal junction

CHAPTER 1 – INTRODUCTION

Artificial insemination (AI) is an established and heavily practiced procedure in the cattle industry, particularly the dairy cattle industry, to assist in intentional pregnancies. Copious notable advantages to artificial insemination include reduction in reproductive diseases or diseases that can spread through reproductive fluids, no maintenance cost of a herd bull, genetically desirable offspring, and partitioning semen to maximize cows serviced with a single ejaculate. AI only/AI use paired with natural service resulted in pregnancy in 87% of US dairy operations compared to 54% in natural service (USDA APHIS, 2018).

In the industry, frozen-thawed semen is preferable over liquid-stored semen because bovine sperm are hardy and the losses due to cryopreservation do not compromise fertility. About 95% of dairy cattle in developed countries that are bred by AI were inseminated with frozen-thawed semen (Chupin & Thibier, 1995). Frozen-thawed semen provides convenient advantages over liquid-stored semen. Cryopreservation extends the shelf-life of semen, allowing time for transportation and testing for transmissible diseases (Bailey et al., 2000). Additionally, viability, motility, and acrosome integrity remain unaffected in post-thaw sperm after long-term storage in liquid nitrogen (Ramírez-Reveco et al., 2016). However, frozen-thawed semen does have limitations. During the freezing and thawing process, extensive and sometimes irreparable damage to the sperm plasma membrane can lead to apoptosis or premature capacitation-like changes (Rodríguez-Martinez, 2000). Likewise, other issues can arise such as chromatin damage, morphological changes, tail abnormalities, and mitochondrial damage (Wooley & Richardson, 1978; Hammadeh et al., 1999). Liquid-stored sperm fortunately are not exposed to such severe damaging conditions but liquid stored sperm have a significantly shorter shelf-life.

After semen deposition occurs through either natural mating or artificial insemination, the sperm will travel through the uterine body, pass the utero-tubal junction, and enter the oviduct. Sperm guidance is primarily facilitated by chemotaxis, thermotaxis, and rheotaxis (Eisenbach & Giojalas, 2006; Bahat et al., 2003; Teves et al., 2006; Kantsler et al., 2014; Miki & Clapham, 2013; Sun et al., 2005; Guidobaldi et al., 2012; Ito et al., 1991). In the most caudal region of the oviduct, the isthmus forms a sperm reservoir, wherein the sperm undergo physiological changes collectively termed capacitation (Visconti et al., 1995a, b; Galantino-Homer et al., 1997; Duncan et al., 1993; Burks et al., 1995; Zaneveld et al., 1991; Aitken et al., 1995; Fraser, 1989; Fraser & Monks, 1990; White & Aitken, 1989; Langlais & Roberts, 1985; Hoshi et al., 1990; Yanagimachi, 1994; Visconti et al., 1998). Prior to capacitation, sperm have the ability to bind to the epithelial lining, creating the sperm reservoir/sperm storage (Fazeli et al., 1999; Gualtieri & Talevi, 2000; Petrunkina et al., 2001). Sperm with epithelial contact have maintained sperm motility and viability longer compared to sperm without epithelial contact (Smith & Yanagimachi, 1990). Sperm binding and subsequent reservoir formation are due to carbohydrate-mediated action between glycans on the oviduct epithelium and candidate lectins on the sperm (Hunter, 1981; DeMott et al., 1995; Suárez, 2002; Lefebvre et al., 1997; Suárez et al., 1998; Suárez, 1998). Lewis^{A} trisaccharide was determined as the glycan motif involved in oviduct-sperm binding in cattle (Suárez et al., 1998).

The overall objective of this project was to determine the effects of a specialized bovine semen extender, FSRD4+, and soluble suLe^{A} glycan at different temperatures on bovine sperm viability and motility. Based on previous work, we speculate sperm viability and motility will be significantly higher with the addition of suLe^{A} and in the FSRD4+ extender compared to sperm without suLe^{A} and in dmTALPC. By finding the most optimal combination, we can determine if

liquid bovine semen can be stored for longer times, making it a more feasible alternative to cryopreservation prior to artificial insemination.

Chapter 2 – LITERATURE REVIEW

The Sperm's Journey through the Female Reproductive Tract and Sperm Binding

Prior to fertilization, sperm undergo an arduous journey through the female reproductive tract, in which considerable sperm loss transpires during their transit. Roughly 1% of the millions or billions of sperm inseminated successfully are transported into the uterine body from natural mating (Harper, 1982). Depending on the species, semen deposition typically occurs in the anterior vagina during natural mating as documented in humans, rabbits, sheep, and cattle. However, in swine, semen deposition is anterior to the cervix or near the uterine body (Hunter, 1981; Roberts, 1986). Similarly, several rodents and horses also bypass semen deposition in the vagina and instead deposit directly into the uterine body. Through chemotaxis, rheotaxis, and thermotaxis in mammals, the fertilizing sperm travel through the reproductive tract and eventually into the oviduct, where they continue towards the oocyte as demonstrated by *in vitro* studies (Eisenbach & Giojalas, 2006; Bahat et al., 2003; Teves et al., 2006; Kantsler et al., 2014; Miki & Clapham, 2013). Through *in vivo* studies, it has been documented sperm transportation to the oocyte is influenced by chemoattractants such as progesterone from cumulus cells and by oviductal contractions and fluid current (Sun et al., 2005; Guidobaldi et al., 2012; Ito et al., 1991).

After passing into the utero-tubal junction (UTJ), sperm will accumulate at the most caudal region of the isthmus and utero-tubal junction in cattle and pigs respectively or throughout the isthmus in mice and horses (Wilmut et al., 1984; Hunter, 1984; Suárez, 1987; Thomas et al., 1994; Figure 1.1). The accumulation is facilitated by sperm storage. Sperm storage has been cited in humans, sheep, cattle, pigs, mice, hamsters, rabbits, horses, and chickens (Pacey et al., 1995b; Hunter et al., 1982; Hunter & Wilmut, 1984; Hunter, 1984;

Suárez, 1987; Harper, 1973; Overstreet et al., 1978; Smith & Yanagimachi, 1991; Bader, 1982; Brillard, 1993). However, sperm storage differs among species, even between mammals. In some mammals, such as cattle and pigs, sperm reservoirs exist in the oviduct, where sperm incubate in the isthmus by binding to the isthmic epithelial lining (Hunter, 1984; Suárez, 1987). In humans, sperm do not bind the endosalpingeal epithelium as persistently as other models and sperm storage is short-term (Pacey et al., 1995b; Murray & Smith, 1997). Rather than storage in the oviduct, chickens store sperm in specialized sperm storage tubules found in the utero-vaginal junction (Brillard, 1993; Bobr et al., 1964; Schindler et al., 1967).

Sperm that successfully survive the retrograde efflux of the cervix, the immune response of the uterus, and the selective regulatory function of the UTJ, eventually enter the oviduct for sperm storage pre-ovulation (Mitchell et al., 1985; Hunter, 1988; Drobnis & Overstreet, 1992; Suárez, 2008). Sperm of certain criteria, such as intact acrosomes and incompletely capacitated, are able to bind to the epithelial lining for the reservoir (Fazeli et al., 1999; Gualtieri & Talevi, 2000; Petrunkina et al., 2001; Figure 1.2). Sperm-oviduct binding in the isthmic epithelium is mediated by oviduct carbohydrates (Hunter, 1981; DeMott et al., 1995; Suárez, 2002; Lefebvre et al., 1997; Suárez et al., 1998; Suárez, 1998). In addition to the sperm-binding moieties, the narrow passages of the lumen and increased mucosal surface augment sperm retention in the isthmus (Suárez, 1998; Suárez et al., 1997; Hunter, 1995).

Aside from protecting sperm, the reservoir also selects for morphologically normal sperm with minimally damaged chromatin (Petrunkina et al., 2001; Fazeli et al., 2004). While bound, sperm with epithelial contact have lengthened sperm viability and motility compared to their luminal counterparts (Smith & Yanagimachi, 1990). Coincubation with oviductal epithelium *in vitro* extends the fertility and motility of bovine sperm (Chian & Sirard, 1995; Pollard et al.,

1991). Additionally, bound sperm become quiescent and have relaxed motility. The oviduct also plays a critical role in controlling polyspermy, to reduce embryo death (Polge et al., 1970; Hunter, 1973; Hunter & Leglise, 1971). The oviduct reduces polyspermy by limited and gradual sperm release, maintaining a low abundance of sperm at the site of fertilization (Hunter, 1996; Hunter, 1973; Hunter & Leglise, 1971).

The carbohydrate-mediated binding between sperm and the oviduct is likely due to interactions between sperm lectins/candidate receptors and oviduct glycans (DeMott et al., 1995; Dobrinski et al., 1996; Suárez, 1998; Suárez et al., 1998). Sperm storage has been observed in hamsters, horses, pigs, and cows (DeMott et al., 1995; Dobrinski et al., 1996; Topfer-Peterson et al., 2002; Green et al., 2001; Kadirvel et al., 2012; Machado et al., 2014; Lefebvre et al., 1997; Suárez et al., 1998; Ignotz et al., 2001).

Role of Glycans in Sperm-Oviduct Binding

In general, glycans are composed of multiple monosaccharides glycosidically-linked together. Sometimes, these glycans can attach to proteins (forming glycoproteins or proteoglycans), lipids (forming glycolipids) (Dwek, 1996). Glycans on glycoproteins can either be classified as N-glycans or O-glycans (Stanley & Cummings, 2009). O-linked glycans are linked to serine and threonine residues in proteins and N-linked glycans are linked to asparagine residues (Tecle & Gagneux, 2015). The roles of glycans include energy generation, metabolism, nutritional storage, cell recognition, and cell adhesion (Varki, 2016). Relating back to cell adhesion, glycoconjugates can be receptors for other cells or pathogens like viruses and bacteria. Cells are enveloped by a glycocalyx, containing several glycoconjugates that branch out from the membrane and have different functional groups (Tecle & Gagneux, 2015; Brandley & Schnaar,

1986). In gametes, the glycocalyx is decorated with molecules that participate in fertilization-specific activities.

As aforementioned, carbohydrate-mediated binding occurs in the oviduct between the epithelium and sperm (DeMott et al., 1995; Dobrinski et al., 1996; Suárez, 1998; Suárez et al., 1998). Specific glycan-lectin interactions exist in multiple species and are largely responsible for sperm storage. Sialic acid and fetuin, which contains sialic acid-bearing termini, decreased sperm binding in oviduct explants (DeMott et al., 1995). Galactose and exposed galactosyl residues on glycoproteins inhibited equine oviduct-sperm binding (Dobrinski et al., 1996). In pigs, early reports indicated that mannosyl-oligosaccharide ligands bind sperm to the oviduct (Topfer-Peterson et al., 2002). More recent reports undertaking a more comprehensive analysis indicated that biantennary 6-sialylated N-acetyllactosamine (bi-SiaLN) and Lewis^X trisaccharide (Le^X) bound to porcine sperm (Kadirvel et al., 2012; Machado et al., 2014). Additionally, Lewis^A trisaccharide on the oviduct was reported to bind bovine sperm (Suárez et al., 1998; Figure 1.3).

In cattle, there is evidence that a seminal plasma protein, PDC-109 (BSP1/2) adhering to sperm binds the LewisA trisaccharide (Ignotz et al. 2001, Gwathmey et al. 2003, Sostaric et al. 2008). BSP1 is a protein secreted by seminal vesicles in cattle (Ignotz et al., 2001; Manjunath et al., 1987). Since sperm are terminally differentiated, they are unable to transcribe proteins, like BSP1. BSP1 is adsorbed onto the sperm when in contact with seminal plasma at ejaculation (Ignotz et al., 2001). During capacitation, some BSPs are lost and another is proteolytically cleaved, possibly contributing to sperm release from the oviduct (Hung & Suárez, 2010).

In this project, soluble glycans are being studied because soluble glycans presumably elicit similar effects in sperm compared to their cell-bound counterparts. The limitation though is soluble glycans may not completely mimic the epithelium and require less affinity to bind to

sperm. Understanding sperm storage in oviducts could drastically improve how liquid-stored semen extenders prolong sperm viability without cryopreservation (McGetrick et al., 2014).

Soluble Glycans

Theoretically sperm interact differently with soluble glycans compared to cell-bound glycans, whether that is because of sterics, binding affinity, or glycan saturation. The soluble glycans used were attached to polyacrylamide chains, wherein each chain had tens to hundreds of glycans bound. This polyacrylamide-glycan chain is an example of a neoglycoprotein, which is a synthetic bioconjugate of a proteoglycan. In Figure 1.4, a simplified version of a neoglycoprotein shows multiple glycans covalently attached to a protein, bovine serum albumin (BSA). Figure 1.5 shows how soluble glycans attached to polyacrylamide chains interact with sperm, resulting in binding.

Capacitation, Hyperactivation, and Acrosome Reaction

Sperm undergo physiological changes necessary for final maturation before oocyte penetration and fertilization. This process is known as capacitation (Chang, 1951; Austin, 1951, 1952). Contact with the oviductal epithelium is a necessary step for capacitation, which has been observed in pigs, sheep, cattle, humans, and horses (Fazeli et al., 1999; Gutiérrez et al., 1993; Guyader & Chupin, 1991; Chian & Sirard, 1995; Kervancioglu et al., 1994). During capacitation, sperm undergo biochemical and physiological changes that prime it for the acrosome reaction (AR). Namely, these changes include protein tyrosine phosphorylation, protein migration on the plasma membrane, reactive oxygen species generation, ion channel activation, increase in intracellular Ca^{2+} levels, intracellular cAMP production, membrane fluidity changes, suppressed

metabolism and motility, and cholesterol removal (Visconti et al., 1995a, b; Galantino-Homer et al., 1997; Duncan et al., 1993; Burks et al., 1995; Zaneveld et al., 1991; Aitken et al., 1995; Fraser, 1989; Fraser & Monks, 1990; White & Aitken, 1989; Langlais & Roberts, 1985; Hoshi et al., 1990; Yanagimachi, 1994; Visconti et al., 1998). Seminal plasma greatly assisted in sperm motility and viability by providing energy for oxidative phosphorylation, by averting premature capacitation and acrosome reaction, and stabilizing the plasma membrane (Poiani, 2006). After sperm undergo sufficient maturation, they release into the oviduct lumen of the isthmus and fertilize the oocyte near the ampullary-isthmic junction.

Sperm release is still a heavily debated topic, but there are plausible proposals to explain how release occurs. It is understood the number of binding sites in the oviduct epithelium remains largely unaffected (Suárez et al., 1991b; Lefebver et al., 1995; Ballie et al., 1997). Therefore, it is likely sperm release is due to changes experienced by the sperm. In this regard, capacitation-related events may assist in sperm release (Smith & Yanagimachi, 1991; Lefebvre & Suárez, 1996). As for bovine, capacitated sperm demonstrated decreased binding to the oviduct and ligand involved in sperm binding (Ignotz et al., 2001; Revah et al., 2000). Understandably, protein modifications or shedding from the plasma membrane of the sperm, in addition to other capacitation changes, could contribute to reduced affinity to the oviduct and subsequent sperm release.

There are other notable modifications sperm experience to assist in sperm release. For example, it has been observed that hyperactivation hastens detachment from the epithelium (DeMott & Suárez, 1992; Pacey et al., 1995a). Hyperactivation is the asymmetrical, high amplitude beating of the flagella, giving it exaggerated motility (Yanagimachi, 1970). In addition to sperm release, hyperactivation is also imperative in the penetration of the zona pellucida

(Stauss et al., 1995). Lastly, hyperactivated sperm are able to traverse through the highly viscous fluids contained in the oviduct (Suárez et al., 1991a; Suárez & Dai, 1992; Quill et al., 2003).

Hyperactivation is not a spontaneous occurrence, but is dependent upon the activation of CatSper, a calcium channel (Ho et al., 2009). Normally, sperm bound to the epithelium experience suppressed motility (Burkman et al., 1984; Overstreet et al., 1980; Overstreet & Cooper, 1975). If the CatSper channel is activated, then Ca^{2+} will flood the sperm from altered membrane potential, resulting in increased intracellular calcium levels, and leading to hyperactivation (Darzon et al., 2009; Quill et al., 2003; Carlson et al., 2003). CatSper is sensitive to extracellular pH increases from oviductal fluid during estrus, which could result in activated CatSper channels and hyperactivation (Suárez 2008a). Bicarbonate levels are the affector of the pH levels by activating adenylate cyclase, increasing cAMP, and increasing pH levels, which activates CatSper (Darzon et al., 2011). A known activator of CatSper in human sperm is progesterone, inducing hyperactivation (Lishko et al., 2011; Ren et al., 2001; Strünker et al., 2011).

Provided that the sperm encounter the oocyte and cumulus cells, they need to be able to acrosome react to penetrate the cumulus cells and zona pellucida before fertilizing the oocyte. To realize this, sperm undergo an exocytotic process known as the acrosome reaction (Yanagimachi, 1994). Bovine sperm are sensitive to progesterone but cannot acrosome react only with progesterone. Bull sperm treated *in vitro* with heparin capacitated more quickly (Lefebvre & Suárez, 1996; Gualtieri et al., 2013). Regardless, acrosome intact sperm are necessary for sperm-oviduct binding and for oocyte penetration and fertilization.

Artificial Insemination and its Practicality in the Cattle Industry

Assisted Reproductive Technologies (ARTs) are procedures done through external manipulation of gametes and embryos to bypass low pregnancy rates or infertility issues. Although technically not considered an ART, artificial/intrauterine insemination (AI) is an instrumental tool that is heavily used in commercial livestock industries, primarily in cattle. In 1990, 70% of US dairy cattle were bred by AI (Hogeland, 1990). From 2005-2014, the percent of US dairy cattle inseminated by AI went from 81%-89% (Khanal & Gillespie, 2013; USDA NASS, 2012; USDA APHIS, 2009; USDA APHIS, 2018).

The advent of AI ushered in new possibilities to meet the demands of the populous. These possibilities are especially true with the introduction of deep-freezing sperm and using cryoprotectants for extended storage (Polge et al., 1949; Lovelock & Bishop, 1959). A survey conducted in 1995 in developed countries showed 95% of all AI in dairy cattle used frozen-thawed semen in dairy cattle (Chupin & Thibier, 1995).

With the success of AI, there are a few benefits that highlight the efficiency of artificial insemination over natural service. Some benefits include reduced herd bull maintenance, reduced disease spread, frequent sperm screening, safety concerns related to handling or natural mating, gene selection, quicker response to detection, and higher pregnancy rates. Typically, AI success is determined by the non-return rate (NRR) and is generally accepted between 65-70% (Vishwanath, 2003). In the US, AI only/AI use paired with natural serviced cows resulted in pregnancy in 87% of dairy operations over natural service at 54% of dairy operations (USDA APHIS, 2018).

Mentioned earlier, sperm are screened for disease as well as sperm quality, typically motility, viability, morphology, and sperm concentration. Semen samples chosen for freezing are

required to have a motility of at least 70% with a concentration of 20 million sperm per ml if freezing in 0.5 mL aliquots (NDDB, 2017). Moreover, post-thaw concentration minimum is 20 million sperm per ml with a motility of at least 50% (NDDB, 2017). A worldwide survey found the average AI dose at 20 million sperm per insemination (Vishwanath, 2003). Interestingly, several authors concluded that the number of sperm needed for AI is between 2.5-5 million per insemination dose (Filseth et al., 1992; van Giesson et al., 1992; den Daas et al., 1998). Even though AI has been a revolutionary change in breeding techniques, there are still questions surrounding in what situations it is best to use either fresh vs frozen semen.

Comparison of Fresh vs Frozen-thawed Semen

As mentioned before, AI is an invaluable tool that has shaped how the cattle industry operates. As such, the dilemma of fresh semen vs frozen-thawed semen is one to review. There are a plethora of concerns when determining which is the most suitable to use, primarily the effects of storage on sperm quality.

Frozen-thawed semen was quickly picked up as an industry standard for cattle with the advent of cryoprotectants such as glycerol and dimethyl sulfoxide and the durable nature of bull sperm (Polge et al., 1949; Lovelock & Bishop, 1959). Semen storage in liquid nitrogen extended storage time considerably compared to frozen carbon dioxide (Haugan et al., 2007). The attraction of frozen semen is mainly convenience. When using frozen-thawed sperm, transportation is not a concern, seeing as how semen can be readily shipped in straws, replacing a herd bull. Cryopreservation leads to genetic improvement and disease control in livestock (Bailey et al., 2000). It has been proposed semen could survive deep-freezing for over 3000 years (Mazur, 1980). Frozen-thawed sperm do not decline in fertilizing capability whether stored in

liquid nitrogen or in dry ice (Vishwanath & Shannon, 2000). Likewise, a study researching the effect of post-thaw quality in bull semen over long-term storage (45 years) noticed no significant differences in acrosome integrity, motility, or viability (Ramírez-Reveco et al., 2016).

There are, however, limitations faced by frozen-thawed semen. Upon thawing, roughly 50-70% of bovine sperm survive (Watson, 1995). Additionally, there is chromatin damage and significant morphological changes, such as mitochondrial damage and sperm tail abnormalities in frozen-thawed sperm (Wooley & Richardson, 1978; Hammadeh et al., 1999). The cooling process is a stressor to sperm by disrupting membrane function and altering membrane permeability, subsequently leading to osmotic stress (Amman & Graham, 1993; Lessar et al., 2000; Mazur, 2004; Kashuba et al., 2014; Meryman, 2007). Cryoprotectants are known to be toxic to sperm, but are necessary for the freezing process to minimize ice crystal formation (Davidson et al., 2014).

Fresh semen also has benefits and limitations. The concentration of sperm needed is significantly less than frozen-semen and allows for a greater number of inseminations in a short period of time (Bucher et al., 2009). Interestingly, very low sperm concentration of liquid bovine semen results in reduced oxidative stress because less sperm are present (Murphy et al., 2013). Comparing fertility of fresh and frozen-thawed semen, several studies confirm no differences in *in vivo* or *in vitro* trials in cattle (Shannon & Vishwanath, 1995; Buckley et al., 2003; Berry et al., 2011; Verberckmoes et al., 2005). A recently studied concern with liquid-stored semen relates to transportation, specifically vibrations affecting sperm quality. The study revealed that vibration, depending on the frequency, caused diminished acrosome and plasma membrane integrity and mitochondrial activity (Schulze et al., 2018). One of the largest drawbacks to liquid-stored semen is the short shelf life (Verberckmoes et al., 2004; Vishwanath & Shannon,

2000). Despite this, fresh semen has relatively inexpensive storage costs and comparatively low insemination concentration compared to frozen semen (Vishwanath & Shannon, 2000; Verberckmoes et al., 2004). Dead sperm influence the viability of the entire collection of semen. As is evident in liquid-stored semen for cows and horses, samples coincubated with additional dead sperm experienced harmful effects such as viability and motility loss (Shannon & Curson, 1972; Brinkso et al., 2003). This observation is not solely unique to liquid-stored semen. In fact, the harmful effects caused by dead cells in pigs were even more extensive in frozen-thawed semen compared to liquid-stored semen (Martinez-Alborcia et al., 2012).

Extenders, whether used in liquid-stored semen or in frozen-thawed semen, are designed to prolong sperm fertility (Vishwanath & Shannon, 2000). There are several kinds of extenders that exist such as egg yolk-based, milk-based, vegetable-based, and soy-lecithin-based (Kasimanickam et al., 2011; Gil et al., 2003; Aires et al., 2003). My research used an egg yolk-based extender. The FSRD4+ extender is presumably similar in content makeup to Caprogen, seeing as how both extenders originated from the same researchers. Caprogen is an egg yolk-based extender. The most optimal temperatures for *in vitro* trials were between 16°C-21°C in Caprogen (Shannon & Curson, 1984). With the advent and improvements of Caprogen, insemination doses decreased from five to two million sperm per insemination (Shannon et al., 1984).

The utilization of liquid-stored is still vital and benefits countries that are small and have fast transportation. It might also have significant benefits to regions that do not have routine access to liquid nitrogen and have poor infrastructure exists (Vishwanath & Shannon, 2000).

Importance of Sperm Quality

Observing and measuring semen quality is a critical component to determining the "best" semen. Sperm attributes that can contribute wholly towards predicting better quality semen are normal morphology, progressive motility, hyperactive motility, acrosomal enzymes, acrosome status, chromatin integrity, stable plasma membrane, and metabolism (Amann & Hammerstedt, 1993). Passage through the cervix reduces the proportion of sperm with tail abnormalities (Karabinus & Saake, 1987). Sperm with poor motility are removed by the female tract shortly after insemination as is evident in the backflow (Hernández-Caravaca et al., 2015). Sperm motility and hypermotility are essential to bypass the folds and crypts of the cervix and oviduct, the cervical mucus, the oviductal fluid, and penetrate the zona pellucida and oocyte.

As alluded to earlier, the UTJ actively filters out morphologically abnormal sperm, while the reservoir selects for morphologically normal sperm (Petrunkina et al., 2001; Fazeli et al., 2004). Conversely, the filtering is not foolproof and defective sperm can penetrate and fertilize the oocyte, causing embryonic death due to developmental issues (Saacke et al., 1988; Saacke, 1994; Hawk, 1988; Hawk, 1987). Morphology is often monitored even if the semen used is for AI. To pass the Bull Breeding Soundness Evaluation (BBSE), less than 20% of sperm should have head defects, and ≥70% sperm should have normal morphology (Barth & Oko, 1989).

Unfortunately, measuring only motility, sperm concentration, and morphology are not indicative of fertility (Gadea et al., 2004). There are several other qualities with which to be concerned, such as viability, chromatin damage/DNA integrity, membrane stability, and acrosome status. Sperm are fully differentiated cells that are highly susceptible to external factors and are unable to defend against pathogenic or immunogenic responses and environmental stimuli. Because of this, sperm viability is sensitive. The viability of sperm has a considerable

impact of fertilization, even when sperm are injected into oocytes using ICSI; fertilization rates were 62.2% from oocytes injected with viable sperm and 16.2% fertilization rate from oocytes injected with nonviable sperm (Poe-Zeigler et al., 1997). Normally, dead sperm are caught in the crypts of the cervix, oviduct, or by the oviduct fluid or cervical mucus. In regards to chromatin structure, sperm with stable chromatin are able to reach the site of fertilization and successfully bind to the zona pellucida (Ardón et al., 2008; Tsəkmakidis et al., 2011). Chromatin abnormalities such as abnormally-shaped nucleus or abnormal condensation of chromatin are correlated with reduced fertility (Johnson, 1997; Ostermeier et al., 2000; Ostermeier et al., 2001). The plasma membrane of the sperm is vital in that it acts as a barrier to the environment, plays a role in capacitation and acrosome reaction, contributes to sperm-oviduct binding, and stores DNA. As an extension, the acrosome is necessary for zona penetration, which is facilitated by hydrolytic enzymes stored in the acrosomal vesicle (Yanagimachi, 1994). In IVF trials, sperm with knobbed acrosome defects were unable to penetrate the zona pellucida (Thundathil et al., 2000). Undoubtedly, employing techniques to carefully monitor the sperm quality would increase fertility by reducing the frequency of abnormalities. Likewise, developing more extensive screening methods for determining better sperm quality should be employed.

Prior to this project, minimal work has been performed focusing on the sole effects of soluble glycans on sperm quality, primarily viability and motility. Based on previous information about sperm storage and lectin-glycan interactions, it is plausible the addition of soluble glycans positively affects sperm by extending the storage lifespan and by preserving sperm motility. Presumably, FSRD4+ is a formulaicly similar predecessor to Carprogen and should be at least an equally competent extender, especially compared to culture media. Therefore, the combination of

glycans and extender should compound and further improve sperm quality than either treatments individually or to the negative controls.

Figures:

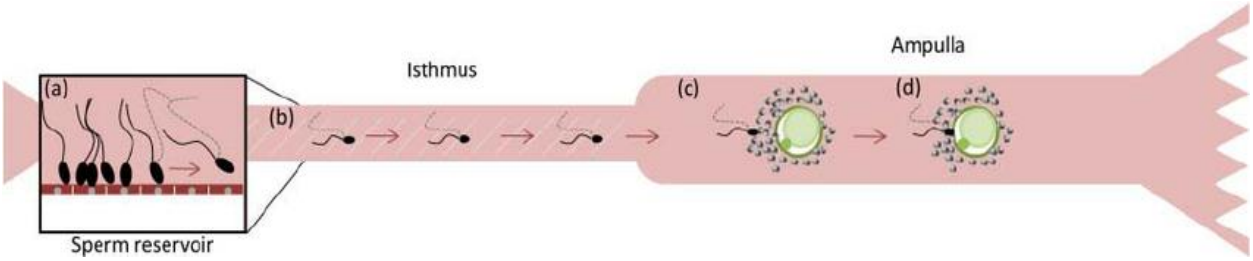

Figure 2.1: A) Sperm binding in the bovine oviduct occurs in the caudal region of the isthmus. B) After final maturation is realized, sperm swim through the isthmus toward the oocyte. C) In the caudal region of the ampulla, sperm penetrate the cumulus cell surrounding the oocyte. D) A sperm cell penetrates the zona pellucida (Coy et al., 2012).

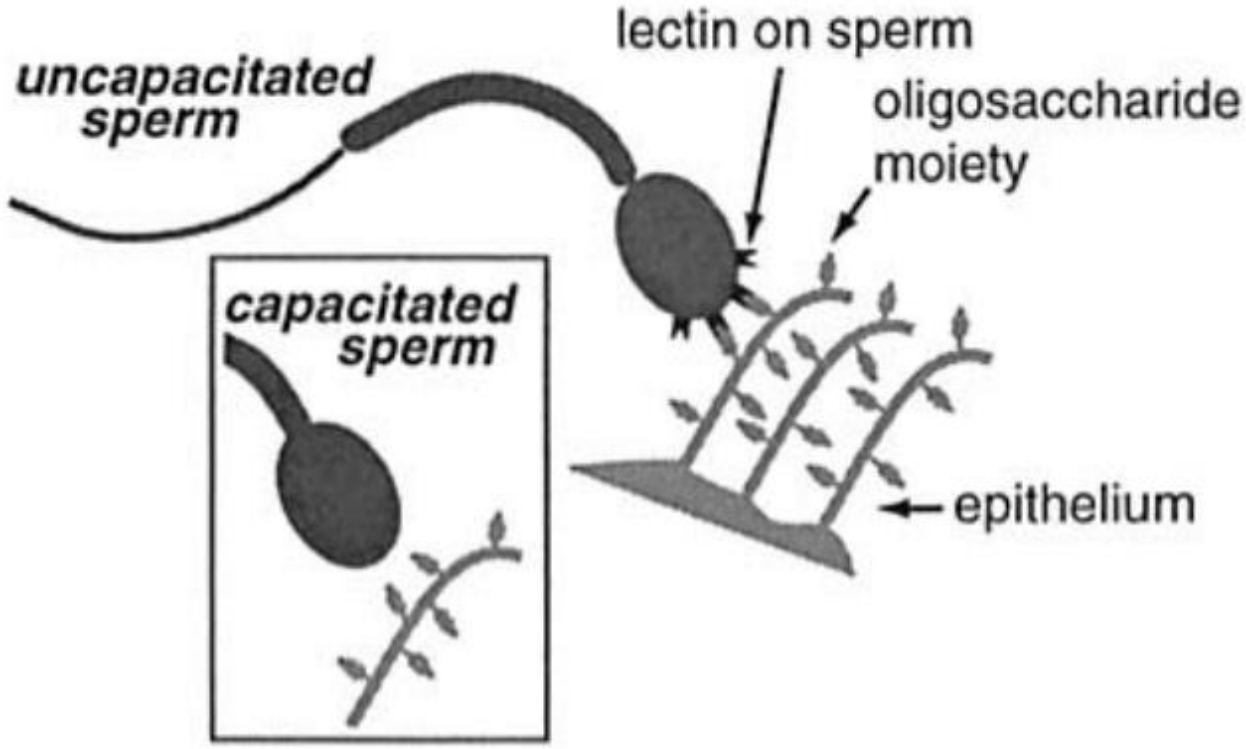

Figure 2.2: Proposed image of sperm reservoir formation. Uncapacitated sperm have lectins on the apical surface of the head, which allows the sperm to bind to the oviduct epithelium by lectin-glycan interactions. Conversely, capacitated sperm are unable to bind to the epithelium (Suárez, 1998).

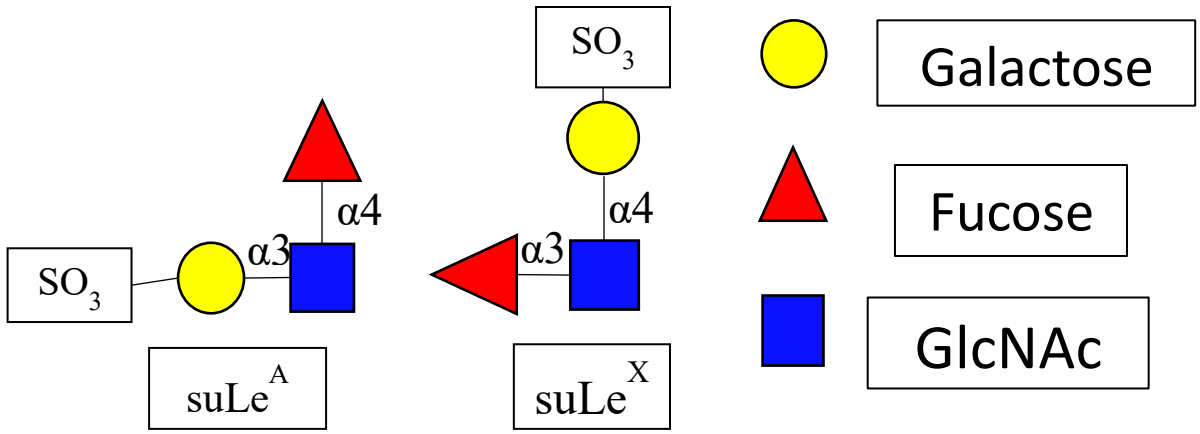

Figure 2.3: Visual representation of suLe^{A} and suLe^{X}. These glycans are positional isomers of one another and have a sulfate group bound to galactose compared to Le^{A} and Le^{X}.

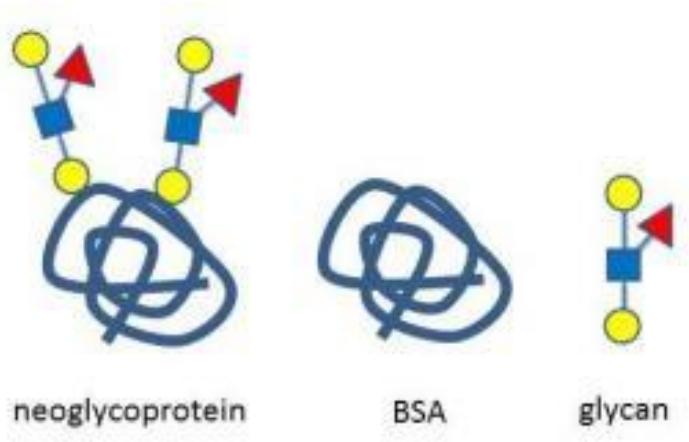

Figure 2.4: Taken from the Elicityl OligoTech® website. Shows an example of a neoglycoprotein made from BSA and a glycan.

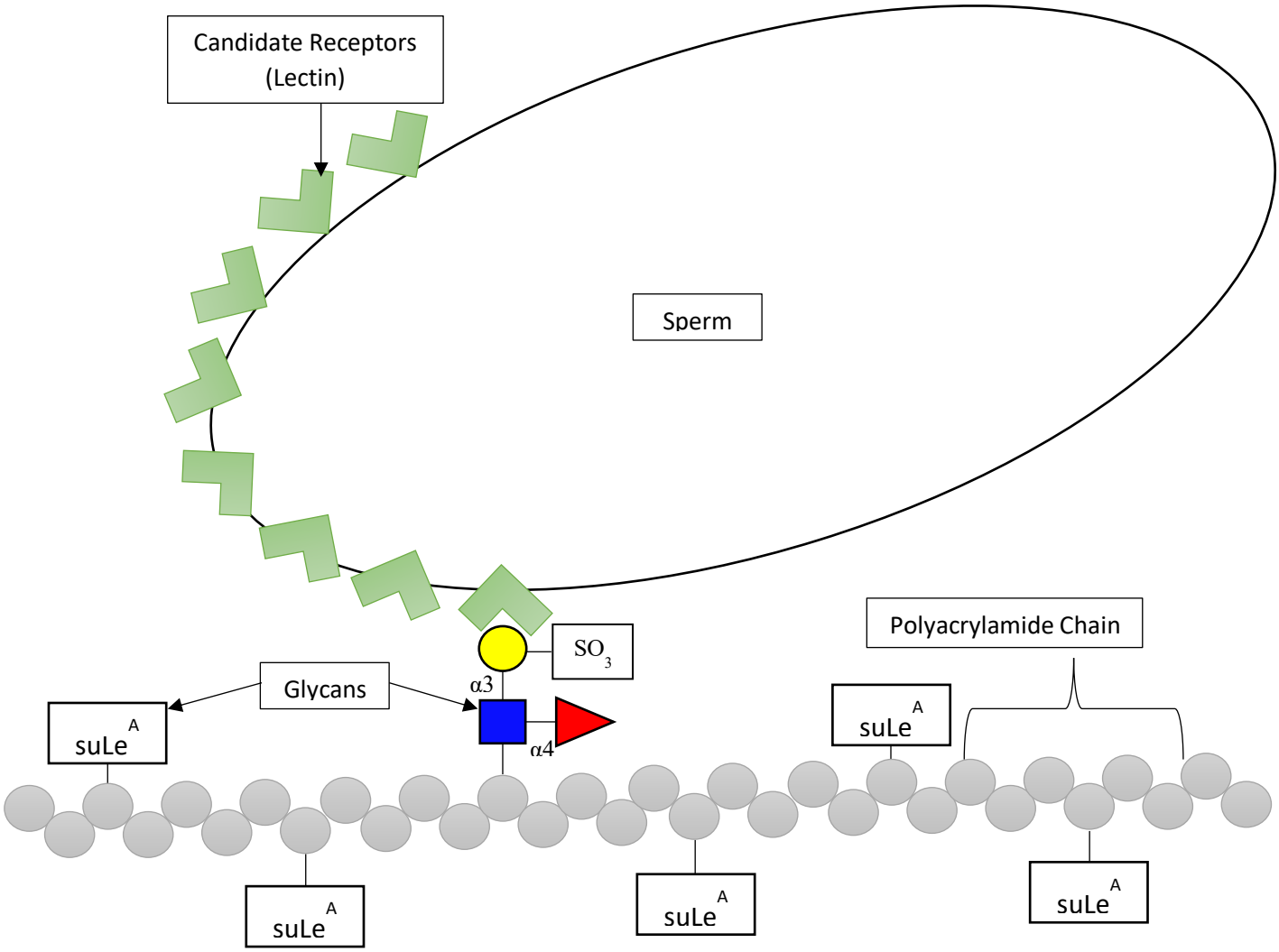

Figure 2.5: Sperm interacting with soluble glycans attached to polyacrylamide chain. A visual representation of a sperm lectin binding to soluble suLe^A attached to a polyacrylamide chain. This image is not to scale.

CHAPTER 3 - THE COMBINATIVE EFFECT OF SOLUBLE suLe^{A} GLYCAN AND SPECIALIZED BOVINE SEMEN EXTENDER, FSRD4+, ON BULL SPERM QUALITY

Materials and Methods

FSRD4+ Preparation

FSRD4+, an experimental egg-yolk-based extender from Sexing Technologies Genetics (Navasota, TX), was prepared at least 24 hours before use. Prior to any extender preparation, all instruments that could be autoclaved were autoclaved. After proper autoclaving, all equipment was wiped with 70% ethanol before being used in a biological safety cabinet. The extender was measured in a 100-mL graduated cylinder as 95% of the total volume needed for extender preparation. The next step was to isolate the egg yolk by separating the white from the yolk and carefully rolling the yolk on disc paper to remove the most superficial layer of egg white without breaking the vitelline membrane. After carefully rolling the yolk, I found an area exposed and pipetted out 5% volume into the graduated cylinder. I poured the contents of the graduated cylinder into an Erlenmeyer flask with a magnetic stir bar. After mixing the solution for 15 minutes using a stir plate at room temperature, I bubbled nitrogen gas for 30 minutes into the solution to replace dissolved oxygen. When finished bubbling, the solution was stored at 4°C for 4 hr to allow sedimentation. The extender was spun in an ultracentrifuge at 9200xg for 1 hour at 4°C. At the end of centrifugation, I pipetted out the supernatant into a clean conical tube and discarded the pellet. I checked the pH of the extender and kept it within the pH range 6.8-7.1. One % (v/v) Gibco Antibiotic-Antimycotic (100x) by Thermo-Fischer Scientific (Waltham, MA) was added to the extender prior to refrigeration at 4°C.

pH Check

Both the FSRD4+ extender and dmTALPC were checked to determine if they were in acceptable pH ranges, which are 6.8-7.1 and 7.3-7.4 respectively. The pH meter used was the Fisherbrand™ accumet™ AB15 Basic by Thermo-Fischer Scientific (Waltham, MA).

Sperm Preparation

Liquid-extended pooled bovine semen from 3 different bulls each collection from Genex Cooperative Inc. (Tiffin, Ohio) was collected. Semen was shipped overnight for immediate use the morning after collection. Three mL of semen, 10 mL of Percoll wash (5.4 mL Percoll, 0.6 mL 10X HBS (1.3 M NaCl, 40 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$), 4 mL dmTALPC), 25 mL of dmTALPC (2.1 mM $CaCl_2$, 3.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.29 mM KH_2PO_4, 0.36% lactic acid, 25 mM HEPES, 10 mM $NaHCO_3$, 0.6% Fraction V BSA, 1 mM Sodium Pyruvate [pH7.3], sterile filtered) for washing, 10 mL of dmTALPC, and 10 mL of the extender FSRD4+ were all warmed to 37°C incubator for 15 min before washing. The 3 mL of semen was gently pipetted on top of the Percoll gradient, wherein the 15-mL conical tube was spun at 796xg for 12 minutes with a tabletop centrifuge. The supernatant was removed by aspiration using a vacuum filter flask, leaving the pellet behind for further washing. The pellet was resuspended in 5 mL of warmed dmTALPC and centrifuged at 196xg for 5 minutes. The supernatant was aspirated again. The pellet was resuspended in 5 mL of warmed dmTALPC and centrifuged again at 196xg for 5 minutes. After the third centrifugation, the sperm pellet was isolated and resuspended in 5 mL of warmed dmTALPC.

Sperm Concentration Measurement

Sperm concentration was measured using the hemocytometer. After washing, an aliquot of sperm was diluted to a 1:100 concentration in deionized water. Ten µL of diluted sperm were loaded onto the top and bottom grooves of the hemocytometer and set for 2 minutes before counting to allow the sperm to settle. Once the average of the top and bottom wells were calculated, the sperm were then diluted to 2.0 million sperm/mL in the FSRD4+ extender and dmTALPC.

Experimental Design

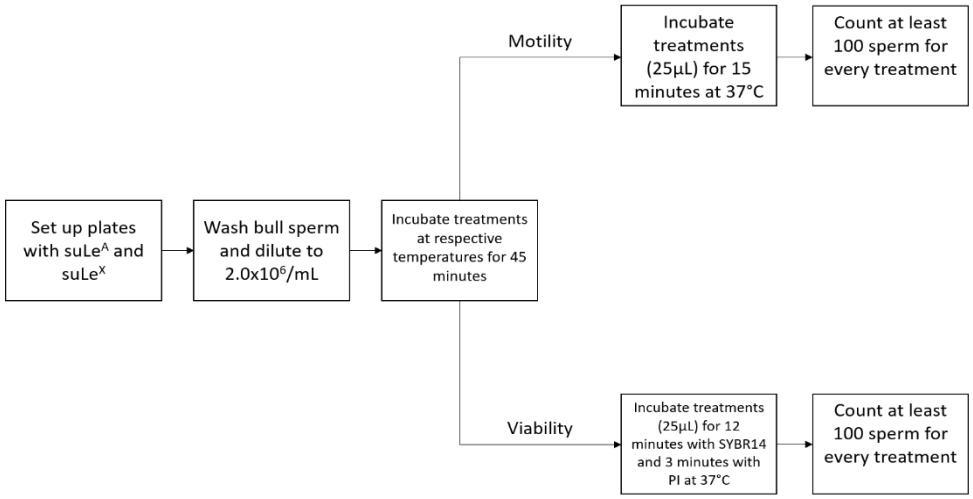

Figure 3.1: Flowchart of experimental design after sperm preparation. From left to right shows a streamlined version of plate setup, sperm concentration, and incubation. After incubation, two experiments are performed to determine viability through fluorescent stains and motility through bright field microscopy.

Soluble Glycan Plate Setup

To slow evaporation and improve the efficiency of the trials, each of the 4 temperature points had their own 96-well plate with 3 soluble glycan treatments, 5 time points, and 2 media types. The treatment types were separated vertically (Y axis), e.g. extender with $suLe^X$, extender with $suLe^A$, extender with no glycans, dmTALPC with $suLe^X$, dmTALPC with $suLe^A$, and dmTALPC with no glycans. The time points were denoted by horizontal labeling (X axis), e.g. 1 hr, 24, hr, 48 hr, 72 hr, and 96 hr. The concentration of soluble glycans in each well was 50 μg/mL. The concentration of glycan used was based on previous work by Kadirvel et al., 2012 with porcine sperm.

Sperm Viability

During the incubation period for the 1-hr time point, fluorescent stains were diluted to 20 μM for SYBR14 and 800 μM for propidium iodide (PI). The stains were obtained from Thermo-Fischer Scientific (Waltham, MA). Twenty-four black 1.5-mL microcentrifuge tubes were labeled with each of their specific treatments (temperature, media, glycan, and time point). At the 45-min mark of the 1 hr time point, 20 μL of incubated sperm were pipetted from the plates into their respective microcentrifuge tubes and then SYBR14 was added to each tube. The 24 microcentrifuge tubes were incubated for 12 min at 37°C. After the SYBR14 incubation, PI was added into each tube and incubated for an additional 3 min at 37°C. Once the fluorescent stains were fully incubated with sperm, the sperm were pipetted out onto warmed slides where 100 sperm per treatment group were assessed for viability. For time points after 1 hr, the sperm were removed 15 min before their incubation ended and pipetted into separate black 1.5mL microcentrifuge tubes. The stains were added and the viability was performed as described

above. Six replicates were performed for each treatment combination, except no-glycan treatments, but decreased over time due to evaporation or microbial contamination. The no-glycan treatment was introduced later in the project and started with 4 replicates.

Sperm Motility

General motility was measured by microscopy instead of using Computer Assisted Semen Analysis (CASA) due to the growth of bacteria or fungi at longer incubated treatments confusing motility determination. At the end of each treatment's time point, 20 μL of sperm was loaded onto a slide and a coverslip placed on top. The overall percentage of motile sperm for each treatment was determined by counting 100 sperm using a Zeiss Axioskop and Zeiss AxioCam 503 mono (Zeiss Microscopy, LLC, Thornwood, NY) at 100X magnification. Similar to the viability experiments, the number of replicates decreased over time from 4 replicates for all treatments.

Statistical Analysis

For statistical analysis of the sperm viability and motility data, we used SAS software v. 9.4 (SAS Institute, Inc., Cary, NC) to run repeated measures PROC mixed model. Blocks were created for each week's trial to account for extraneous errors. The model is y=media|glycan|temp|time. Results are presented as least squared means ± SEM. Difference were considered to be significant at $p \leq 0.05$ using Difference of Least Squares Means.

Results

The focus of this project was centralized around the effects of the four treatment groups interacting with each other and how they affect viability and motility of liquid-stored bovine semen. The treatment groups are temperature (5°C, 15°C, 22°C, 37°C), glycans ($suLe^A$, $suLe^X$, no glycan), media (FSRD4+ and dmTALPC), and time (1, 24 , 48 , 72 , 96 , and 120 hr). The temperatures were selected as follows: 5˚C is the approximate temperature of a refrigerator, 15˚C is around the lower temperature range where membrane fluidity transitions between solid and liquid phase (Holt & North, 1984; Holt & North, 1985; Canvin & Buhr, 1989; Crowe et al., 1989) and is around the chilled temperature semen is stored at, 22 ˚C is the approximate room temperature, and 37˚C is the approximate cow body temperature. dmTALPC was the standard used to compare the effectiveness of FSRD4+, and it is somewhat similar to oviductal fluid, but is primarily used as a balanced salt solution. The time points were selected to determine the initial effect (1 hr) treatments had on viability and motility and the subsequent effects at consistent time points (24-120 hr). The viability of bovine semen was checked using SYBR14 and propidium iodide (PI) at their respective time points and recorded as a percentage. Live sperm can exclude PI and accumulate staining with SYBR14. Dead cells do not accumulate SYBR14 staining but cannot exclude PI. In uncommon instances, all moribund sperm, sperm fluorescing green and red, were counted as dead.

Effects on Sperm Viability

Sperm viability was significantly higher during incubation in FSRD4+ extender (66%) compared to dmTALPC (49%) (Figure 3.2). Sperm incubated with $suLe^A$ (61%) had significantly higher viability than sperm without glycans (54%), but viability was not different

between sperm incubated with suLe^A or suLe^X (58%). The viability of sperm incubated with suLe^X was no different than the no-glycan control (Figure 3.3). Sperm incubated at 15˚C (66%) and 22˚C (65%) had significantly higher viability than sperm incubated at 5˚C (59%) and 37˚C (41%), but there was no difference between 15˚C and 22˚C (Figure 3.4).

Comparing the media and soluble glycan treatments, suLe^A in FSRD4+ extender (69%) resulted in significantly higher viability compared to all other treatments, except to suLe^X in FSRD4+ extender (66%), which performed similarly (Figure 3.5). When tested at four temperatures, bovine sperm had a significantly higher viability in FSRD4+ extender at 5°C, 15°C, and 22°C (73%, 74%, and 70%) compared to sperm in extender at 37°C (50%) and any treatment in dmTALPC (Figure 3.6). For the glycan and temperature interactions, sperm viability was significantly higher for treatments with suLe^A at 15°C and 22°C (68% and 69%) than all other treatments, except for treatments with suLe^X at 15°C and 22°C (67% and 67%), which performed similarly (Figure 3.7). Bovine sperm viability was significantly higher, around 75-80%, at 1 and 24 hr in FSRD4+ extender compared to dmTALPC at every time point (Figure 3.8). Both suLe^A and suLe^X performed similarly to each other, but they were significantly better than the no glycan treatment at nearly every time point (Figure 3.9). The viability at 1 hr for 15°C (77%) was significantly higher than all other treatment except 1 hr for 5°C and 22°C (74% and 76%) and 24 hr at 15°C (71%), which performed equally as well. Unsurprisingly, viability was significantly highest at 1 hr compared 24 hr-96 hr. Overall, the viability at 15°C and 22°C was significantly better than 5°C and 37°C at each time point and had overall prolonged viability (Figure 3.10).

Effects on Sperm Motility

Sperm motility was determined by counting the total number of sperm exhibiting any movement, including progressive and nonprogressive motility, divided by the number of sperm observed and recorded as a percentage. When comparing overall means, FSRD4+ (26%) yielded significantly higher motility than dmTALPC (11%) (Figure 3.11). Motility was significantly higher when incubated with $suLe^X$ (22%) compared to $suLe^A$ (21%) and no-glycan (13%), and $suLe^A$ compared to no-glycan (Figure 3.12). Over the 120 hr, 15°C storage (30%) yielded significantly more motile sperm than 5˚C, 22°C, and 37˚C (13%, 22%, and 10%), despite it having significantly lower motility than 22°C and 37˚C at the 1 hr time point (Figure 3.13).

In terms of glycans and medium, motility was significantly higher for sperm in FSRD4+ extender with $suLe^X$ (32%) than all other treatments. $suLe^A$ in extender (29%) outperformed all of the dmTALPC treatments and had significantly greater motility than sperm in FSRD4+ extender without any glycans (17%) (Figure 3.14). Sperm motility at 15°C in FSRD4+ extender (43%) was significantly higher than all other temperatures in extender and in dmTALPC (Figure 3.15). Sperm motility was significantly higher in media with $suLe^A$ and $suLe^X$ at 15°C (33% and 35%) than 5°C, 22°, and 37°C with both glycans and all four temperatures without any glycans (Figure 3.16). Motility was significantly higher at 1 hr for both FSRD4+ extender (62%) and dmTALPC (43%) than the subsequent time points. However, sperm motility was preserved better and significantly higher compared to mirrored time points in dmTALPC (Figure 3.17). Reviewing the effect of glycans on sperm motility over time, 1 hr with $suLe^A$ (55%) or $suLe^X$ (57%) was significantly higher than 24-120 hr, and they were significantly higher at all time points than treatments without glycans. In general, sperm motility was fairly similar for $suLe^A$ and $suLe^X$ except for 48 hr and 72hr. They were, however, significantly higher than treatments

without glycans (Figure 3.18). Although motility was significantly higher at 22°C and 37°C at 1 hr (62% and 57%) compared to 15°C (53%), sperm motility was preserved better over time at 15°C compared to other temperatures (Figure 3.19).

Discussion

AI is used abundantly in the dairy cattle industry, especially in developed countries. Most often, AI uses frozen-thawed semen due to convenience, although sperm are damaged during the freezing and thawing process. This study compared a new long-term extender to culture medium, with and without added oviduct glycans, at different temperatures over 96 hr to determine the most optimal approach for sperm storage without cryopreservation.

In reference to viability, FSRD4+ significantly outperformed dmTALPC at every temperature, time point, and glycan treatment. Sperm stored in FSRD4+ maintained higher viability than sperm in dmTALPC. On average, viability in the extender was 66% compared to 49% viability for sperm in dmTALPC. At time point 96 hr, viability in FSRD4+ was 53%, which was similar to the 50-70% viability of sperm after thawing following traditional cryopreservation (Watson, 1995). Overall, addition of $suLe^A$ and $suLe^X$ to sperm resulted in average viability of 61% and 58% respectively, which was higher than sperm with no added glycans (54%). When glycans were added to FSRD4+ extender, the viability of sperm with $suLe^A$ and $suLe^X$ was 70% and 66% respectively. FSRD4+ and $suLe^A$ independently significantly improved the viability of sperm, but when combined, further increased viability, making sperm viability similar to viability of cryopreserved sperm. Factoring in temperature and time, the best combination of three treatments over 96 hr is FSRD4+ extender, $suLe^A$, at 15˚C. Viability from 1 hr to 96 hr dropped 14%, ending at 68%.

In reference to motility, FSRD4+ also significantly outperformed dmTALPC at every temperature, time point, and glycan treatment. The average percentage of motile sperm in FSRD4+ and dmTALPC was 26% and 11% respectively over all times and treatments. The overall average motility of sperm with $suLe^A$ and $suLe^X$ was 21% and 22% respectively. Motility for both glycans in FSRD4+ was roughly 30%, which was significantly better than sperm in extender without glycans. With time and temperature introduced, sperm in 15˚C, with $suLe^X$ or $suLe^A$, and in FSRD4+ had significantly higher motility than other combinations with an ending motility of 20% at 120 hr.

In agreement with Murphy et al., 2014, 15˚C was the best of the four temperatures tested for long-term storage in terms of viability or motility. Extended storage of liquid bovine semen temperatures above 32°C results in decreased progressive motility and membrane integrity; however, storage from 5-22°C had better sperm quality (Murphy et al., 2014). Membranes are easily influenced by external factors, and the behavior of a membrane is largely affected by temperature. Depending on the temperature, membranes can undergo phase transitions (Mantsch & McElhaney, 1991). Higher temperatures result in more fluidity, allowing for lipids to move freely, while lower temperatures result in more rigidity known as a gel phase. Depending on the species, transition temperatures have been reported to ranging from 17-28˚C (Holt & North, 1984; Holt & North, 1985; Canvin & Buhr, 1989; Crowe et al., 1989). It is reasonable to assume sperm had the best motility and viability at 15˚C and 22˚C because these temperatures fell within the thermotropic transition phase, allowing for a semi-structured membrane. As for 5˚C and 37˚C, sperm performed significantly worse. When considering temperature and medium, it is evident FSRD4+ performs well at 15˚C and 22˚C. This is possibly due to cholesterol from the egg yolk of the FSRD4+ extender because it modifies the membrane phase behavior. Cholesterol

increases membrane fluidity below phase transition temperatures, and it decreases membrane fluidity above phase transition temperatures (Wolkers et al., 2002; Oldenhof et al., 2015).

FSRD4+ outperformed dmTALPC in nearly every one-way and two-way interactions. There are number of reasons to speculate why extender use led to significantly better sperm quality. Like many other liquid extenders, FSRD4+ presumably contains antioxidants to reduce ROS production, especially with ample nutrients to support long-term storage. As mentioned before, ROS negatively alter plasma membrane structure and can lead to DNA damage and cellular death. Relating back to nutrient supplementation, both media have salts, sugar, and pH buffers. However, FSRD4+ could have multiple sugars, such as glucose, fructose, sucrose, or trehalose, to provide nutrients or confer protection for prolonged longevity. Additionally, the extender has cholesterol, which allows for a wider phase transition, which allows the sperm to withstand a larger temperature range with minimal effects to the plasma membrane.

Interestingly, sperm with added suLe^{A} and suLe^{X} performed similarly regardless of temperature, time, or media. This was surprising because bovine sperm bind immobilize suLe^{A} with higher affinity than suLe^{X}. The effect of soluble suLe^{X} may be due to a lower affinity threshold necessary for soluble glycans to bind cells, compared to insoluble glycans. The high force required to tether a sperm means that if glycans are insoluble (attached to epithelial cells or bound to beads, for example), only high affinity interactions can be maintained (Curtis et al. 2012). But since the glycans we used are soluble and lack any attachment to oviduct epithelial cells or beads, which can act as anchors, lower affinity interactions can still occur. Our results imply that the low affinity interactions still have some effects on sperm behavior, maintaining motility and viability more effectively than sperm without glycans.

More questions surrounding glycans and glycan interactions still exist. The sulfated versions of glycans Le^A and Le^X are believed to bind sperm with higher affinity (Kadirvel et al., 2012) and are expected to affect sperm function more than the non-sulfated glycans. Sulfated glycans may bind a higher proportion of sperm and have more significant affects on sperm viability and motility.

Three-way interactions between the variables tested were found; however, all three-way interactions that existed were rare and primarily due to time. For example, sperm viability in FSRD4+ with $suLe^A$ at 24 hour was significantly higher than in dmTALPC with $suLe^X$ at 96 hour. There are no explanations as to why these interactions exist and they do not make biological sense. No four-way interactions were observed.

Further experiments monitoring acrosome status, chromatin structure, and ROS production should be performed to determine whether these sperm characteristics are influenced by oviduct glycans and specialized extenders. Based on the overall objectives outlined, this study demonstrated that addition of soluble glycans, particularly $suLe^A$, improves motility and viability compared to sperm incubated without glycans. Moreover, FSRD4+ also increased sperm viability and motility compared to dmTALPC. Although the effects on sperm fertility are not known, these results provide a basis for investigating modifications of storage systems that may lengthen the time that sperm may be stored in liquid form prior to AI.

Acknowledgements

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and shipped bovine semen needed for the experiments. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2015-67015-23228 from the USDA National Institute of Food and Agriculture.

Figures:

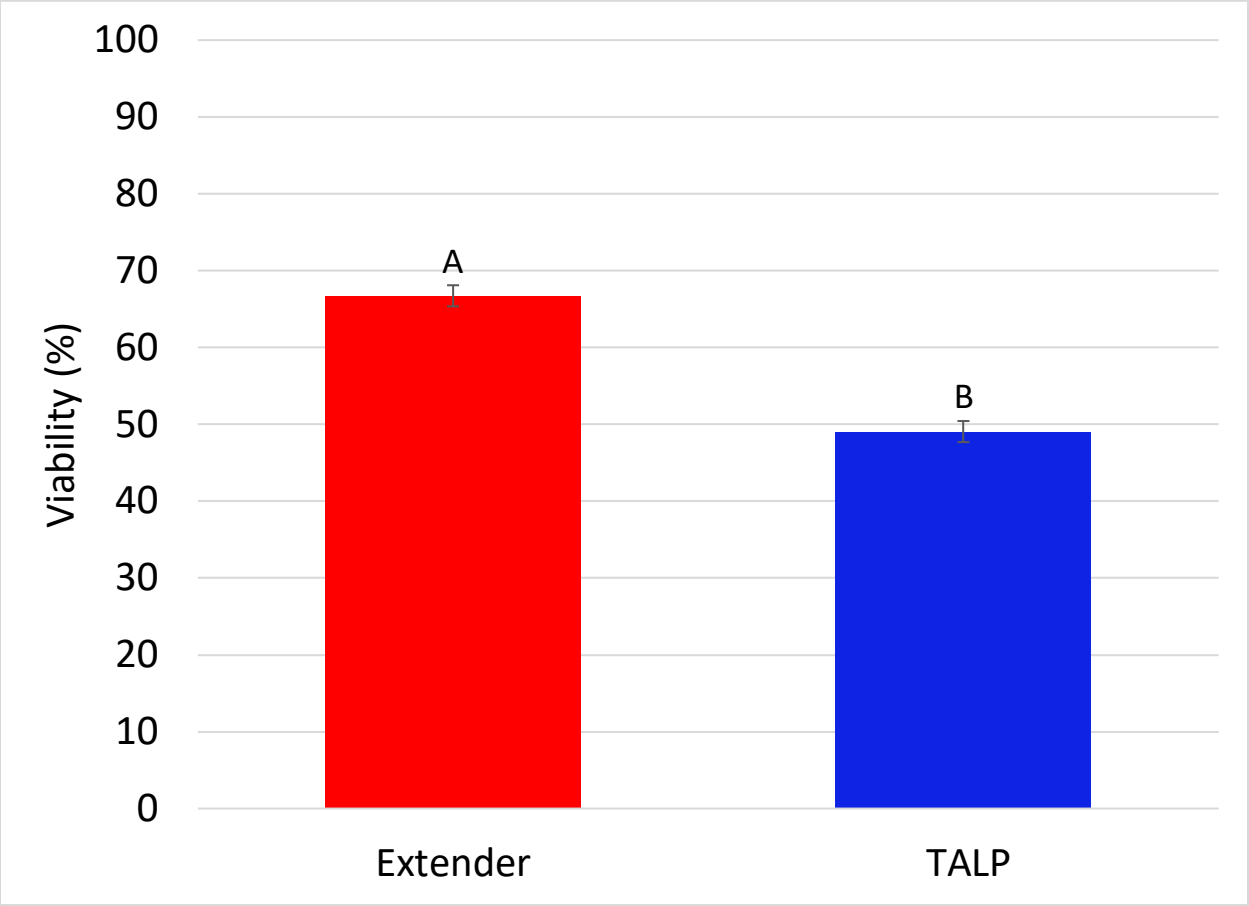

Figure 3.2: Effect of medium on viability. Means averaged over time, temperature, and with and without glycans are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

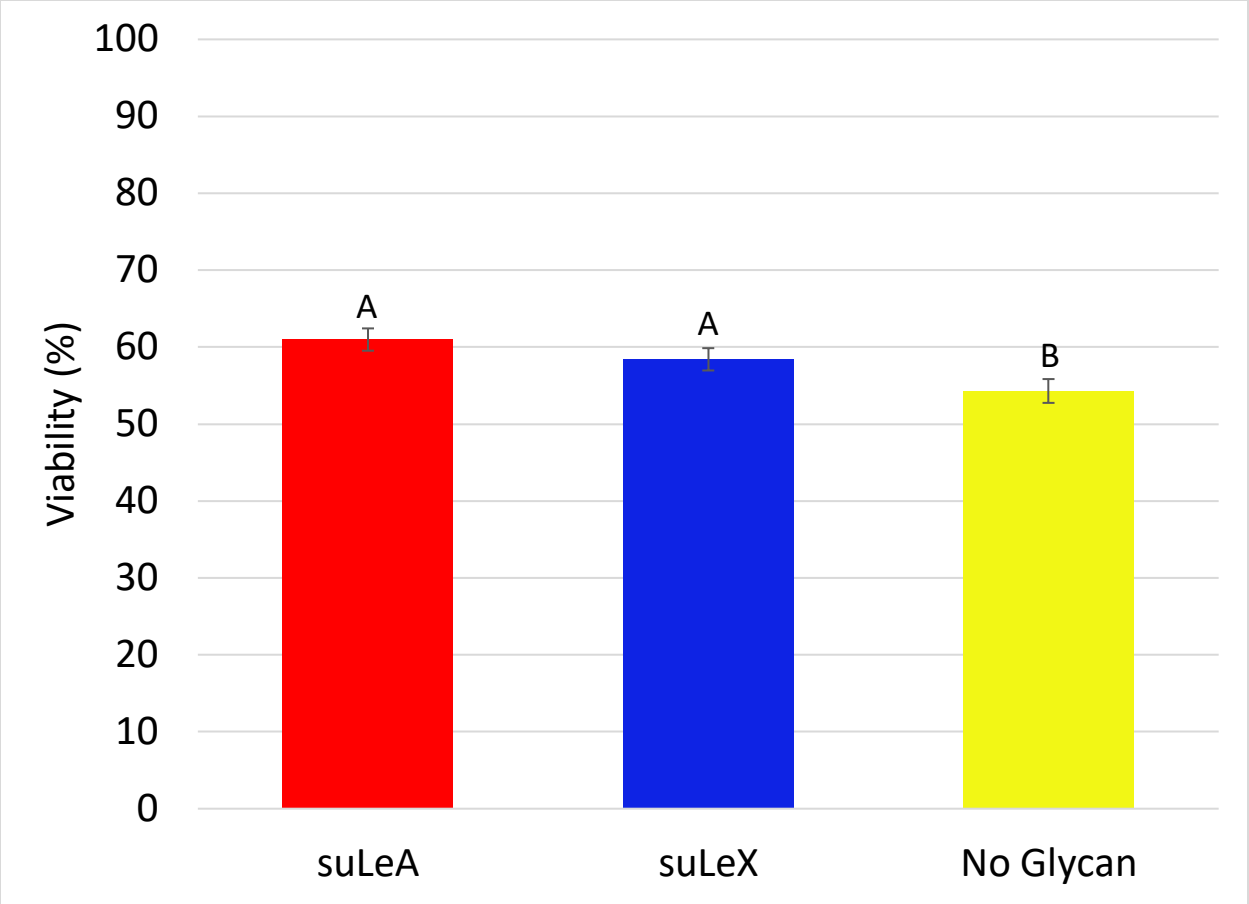

Figure 3.3: Effect of glycans on sperm viability. Means averaged over time, temperature and medium are shown. Means with different letter are significantly different ($p \leq 0.05$). The glycans used were $suLe^A$ (red), $suLe^X$ (blue), and no glycan (yellow).

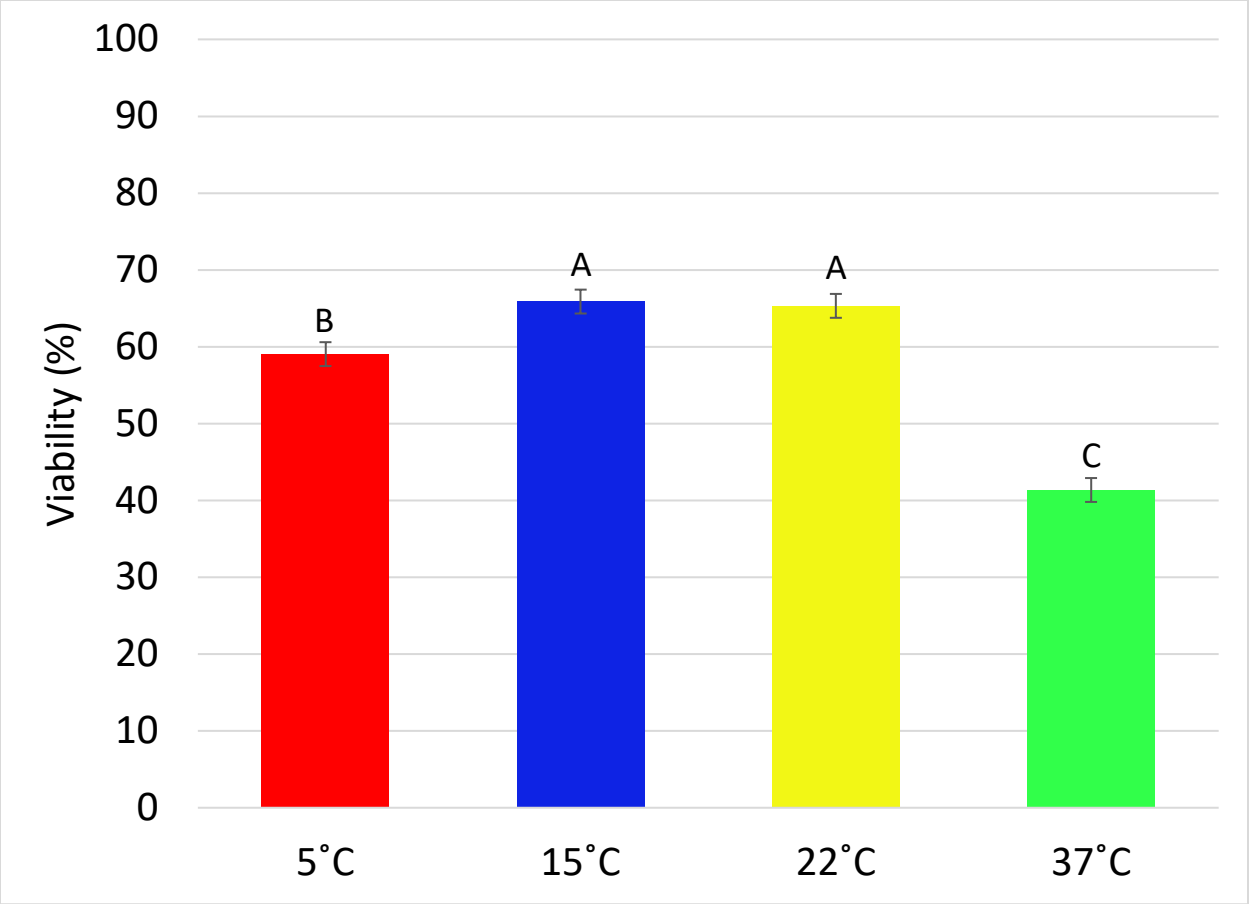

Figure 3.4: Effects of temperature on sperm viability. Means averaged over time, with and without glycans, and media are shown. Means with different letters are significantly different ($p \leq 0.05$). The temperatures used were 5°C (red), 15°C (blue), 22°C (yellow), and 37°C (green).

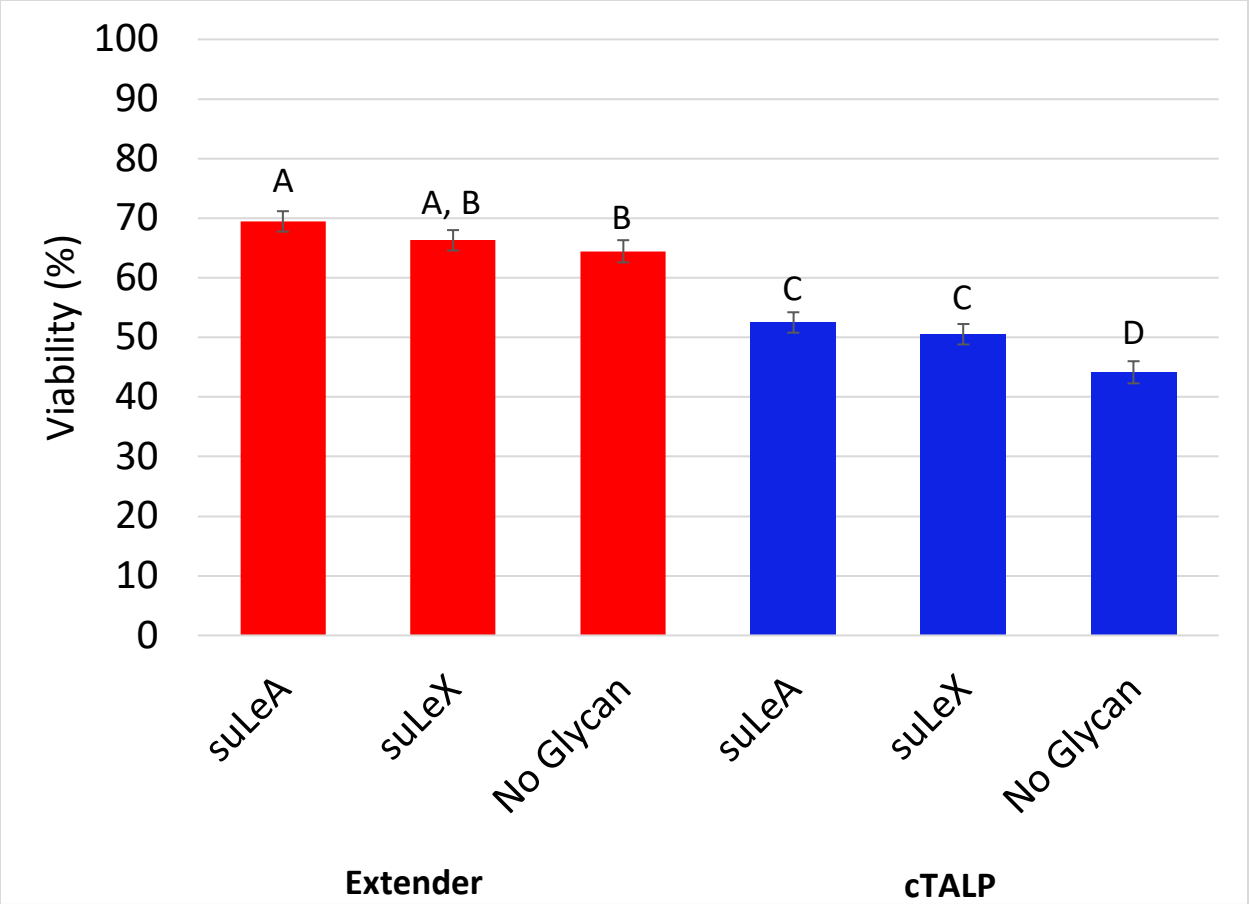

Figure 3.5: Effects of medium and glycan on sperm viability. Means averaged over time and temperature are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

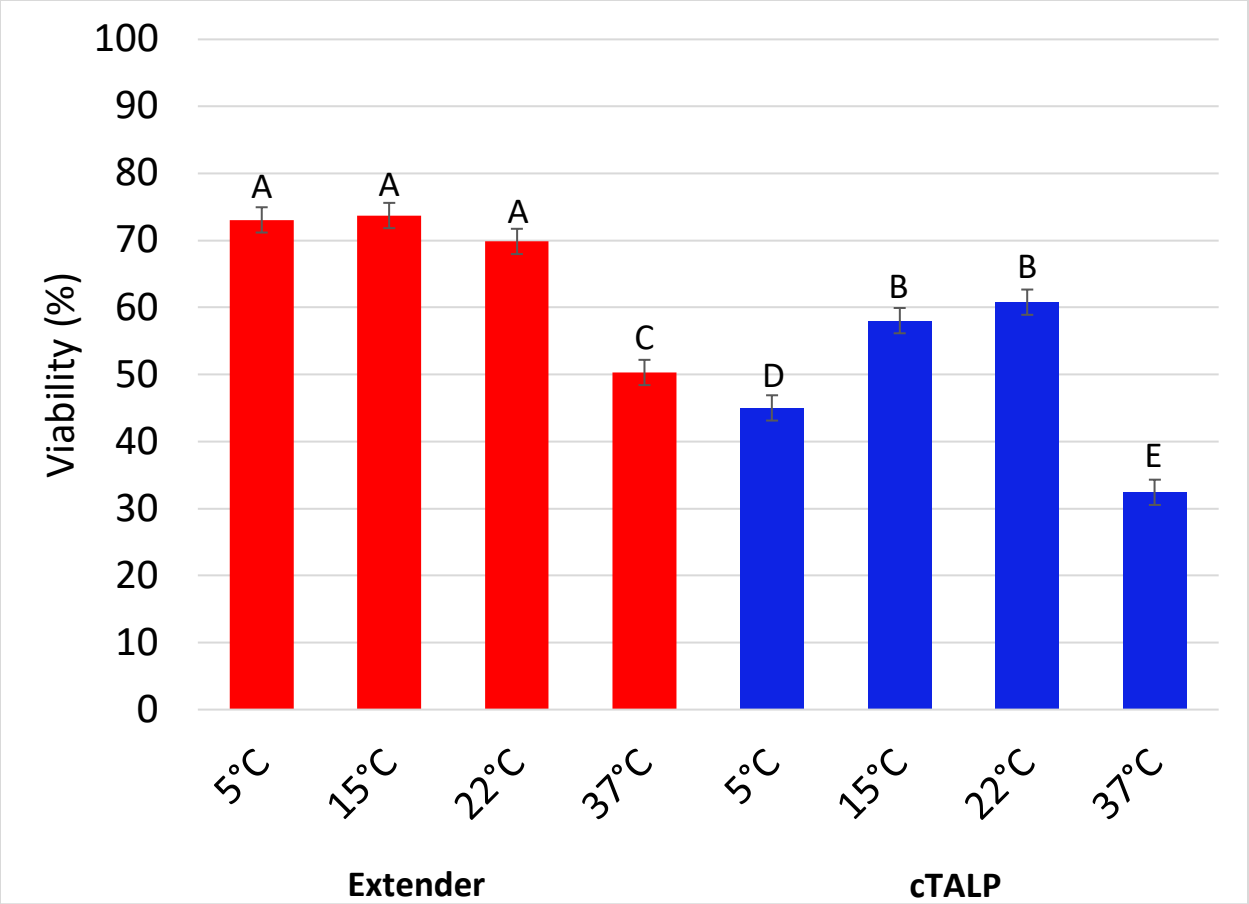

Figure 3.6: Effects of medium and temperature on sperm viability. Means averaged over time and with and without glycans are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

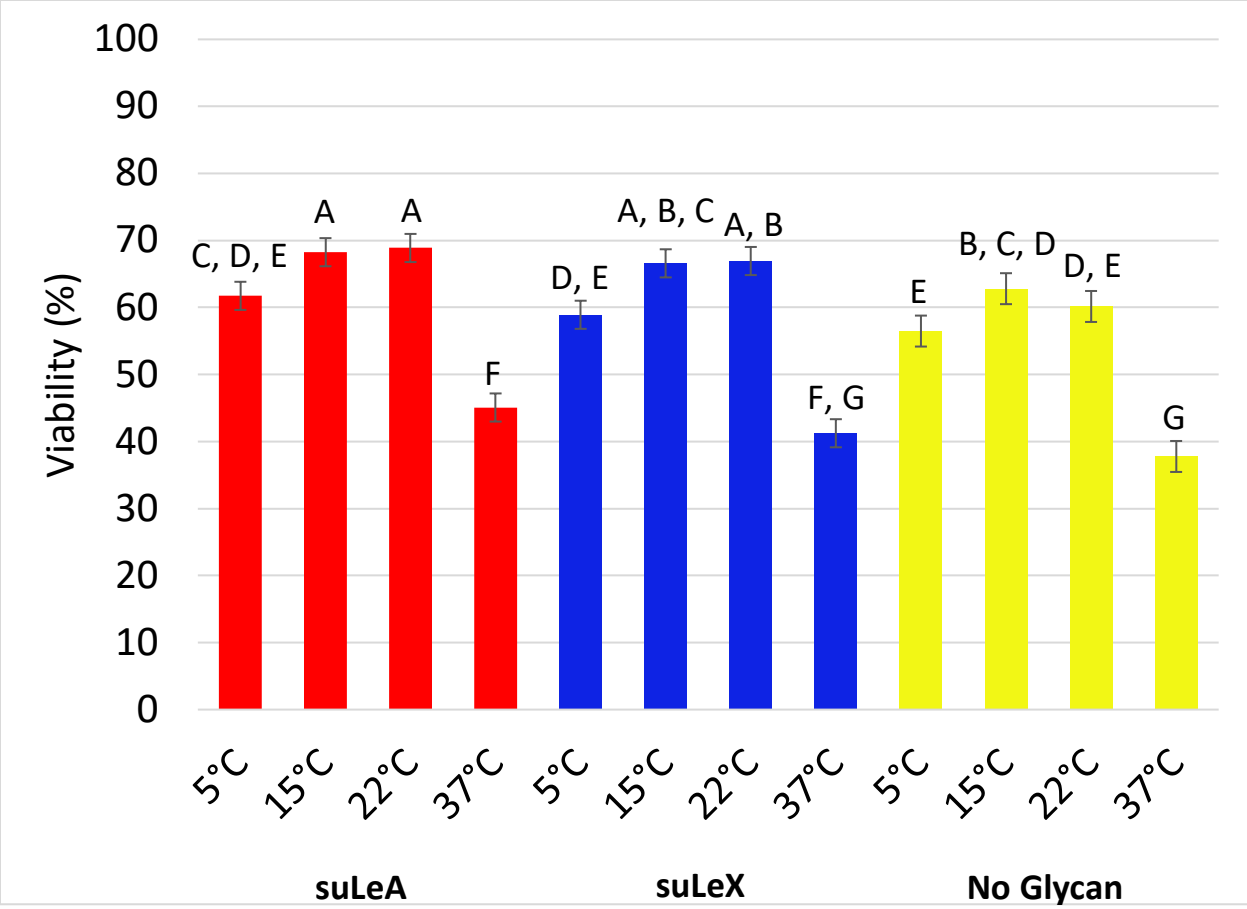

Figure 3.7: Effects of temperature and glycan on sperm viability. Means averaged over time and medium are shown. Means with different letter are significantly different ($p \leq 0.05$). The glycans used were $suLe^A$ (red), $suLe^X$ (blue), and no glycan (yellow).

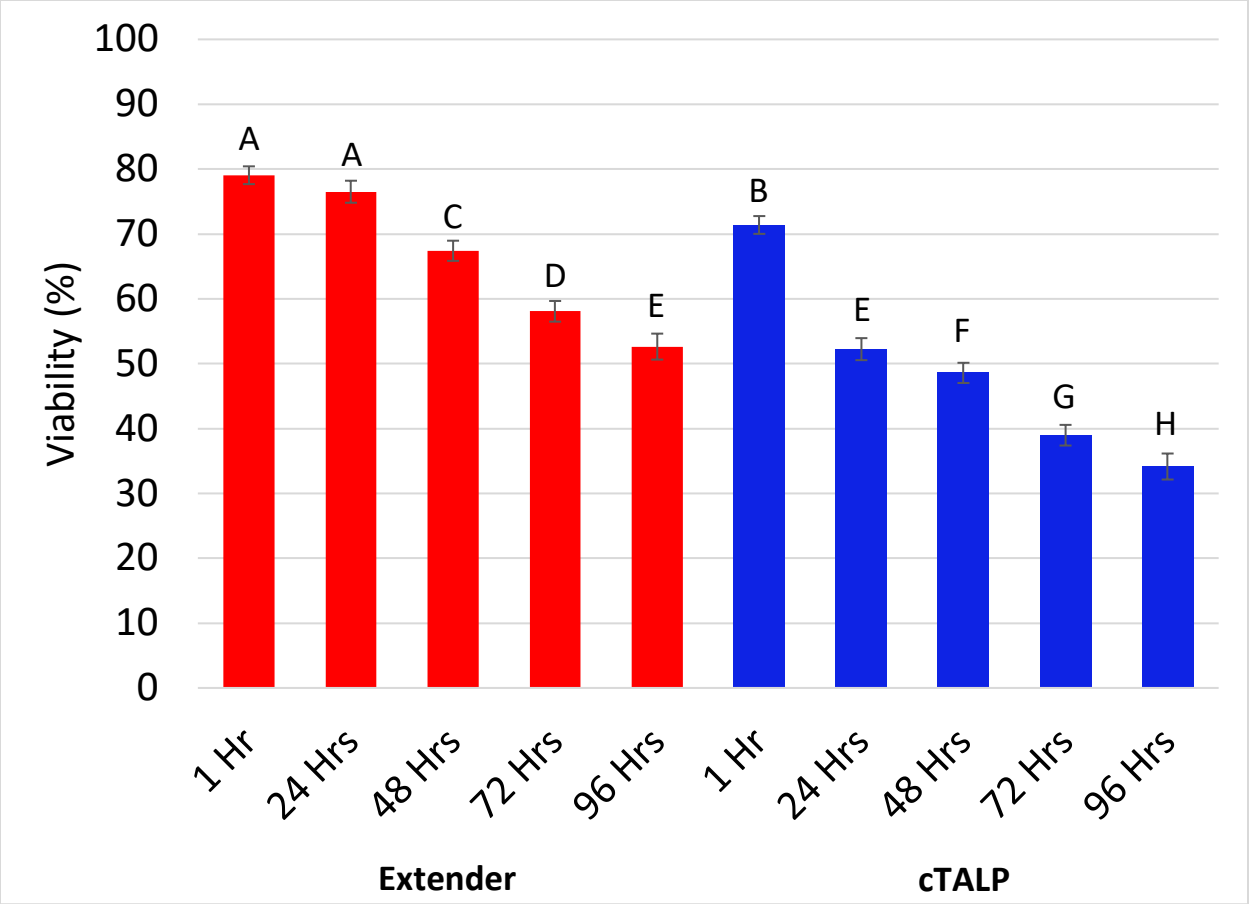

Figure 3.8: Effects of media and time on sperm viability. Means averaged over temperature and with and without glycans are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

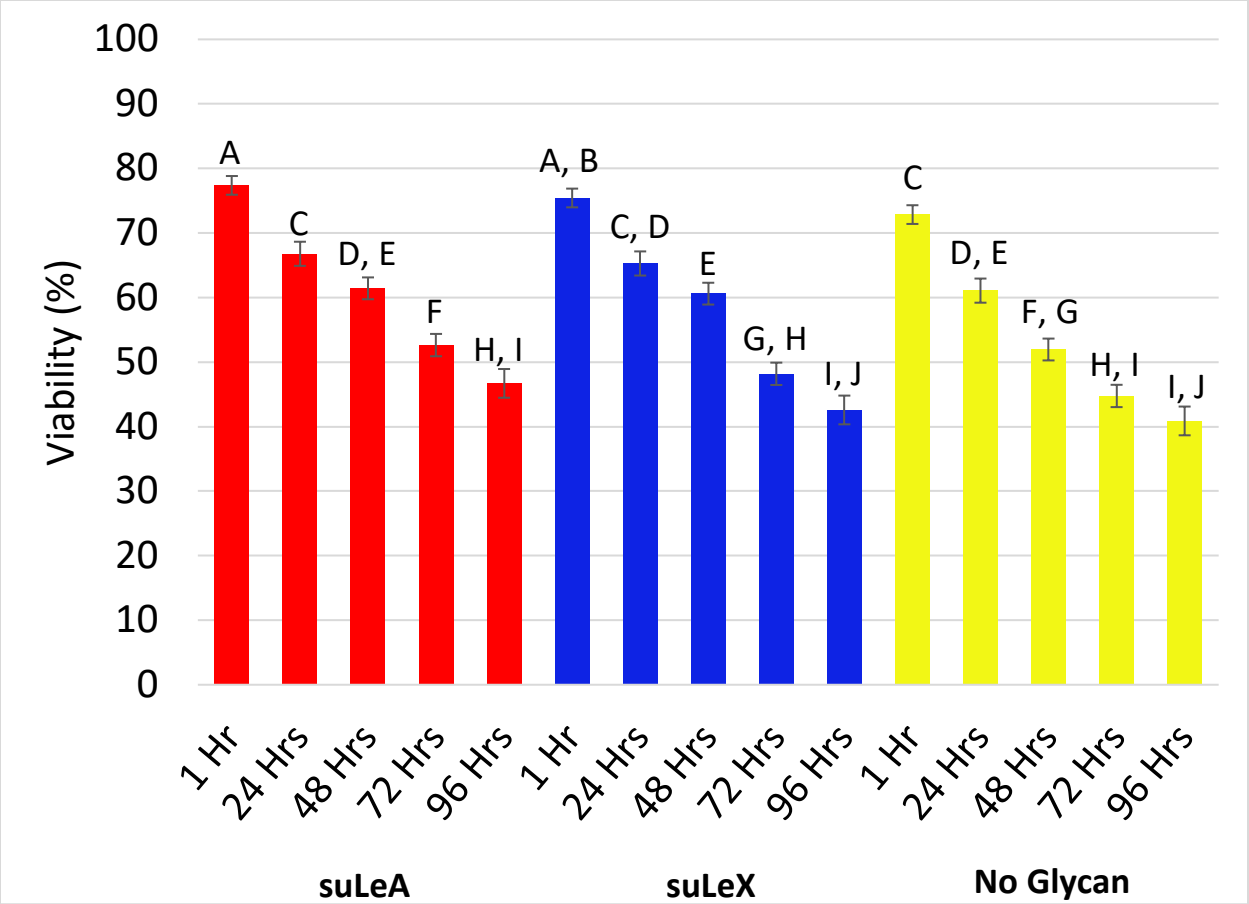

Figure 3.9: Effects of glycan and time on sperm viability. Means averaged over temperature and medium are shown. Means with different letter are significantly different ($p \leq 0.05$). The glycans used were $suLe^A$ (red), $suLe^X$ (blue), and no glycan (yellow).

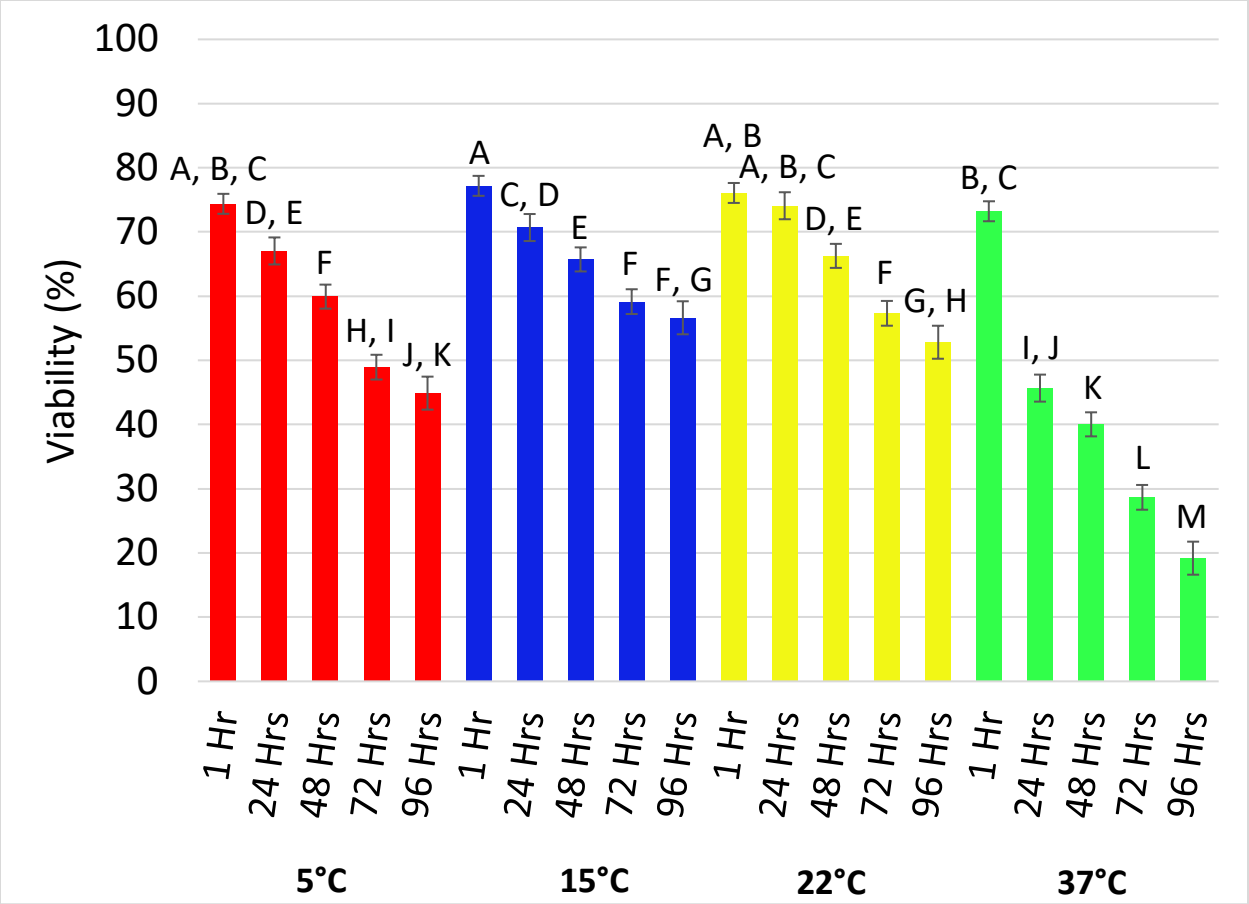

Figure 3.10: Effects of temperature and time on sperm viability. Means averaged over with and without glycans and medium are shown. Means with different letters are significantly different ($p \leq 0.05$). The temperatures used were 5°C (red), 15°C (blue), 22°C (yellow), and 37°C (green).

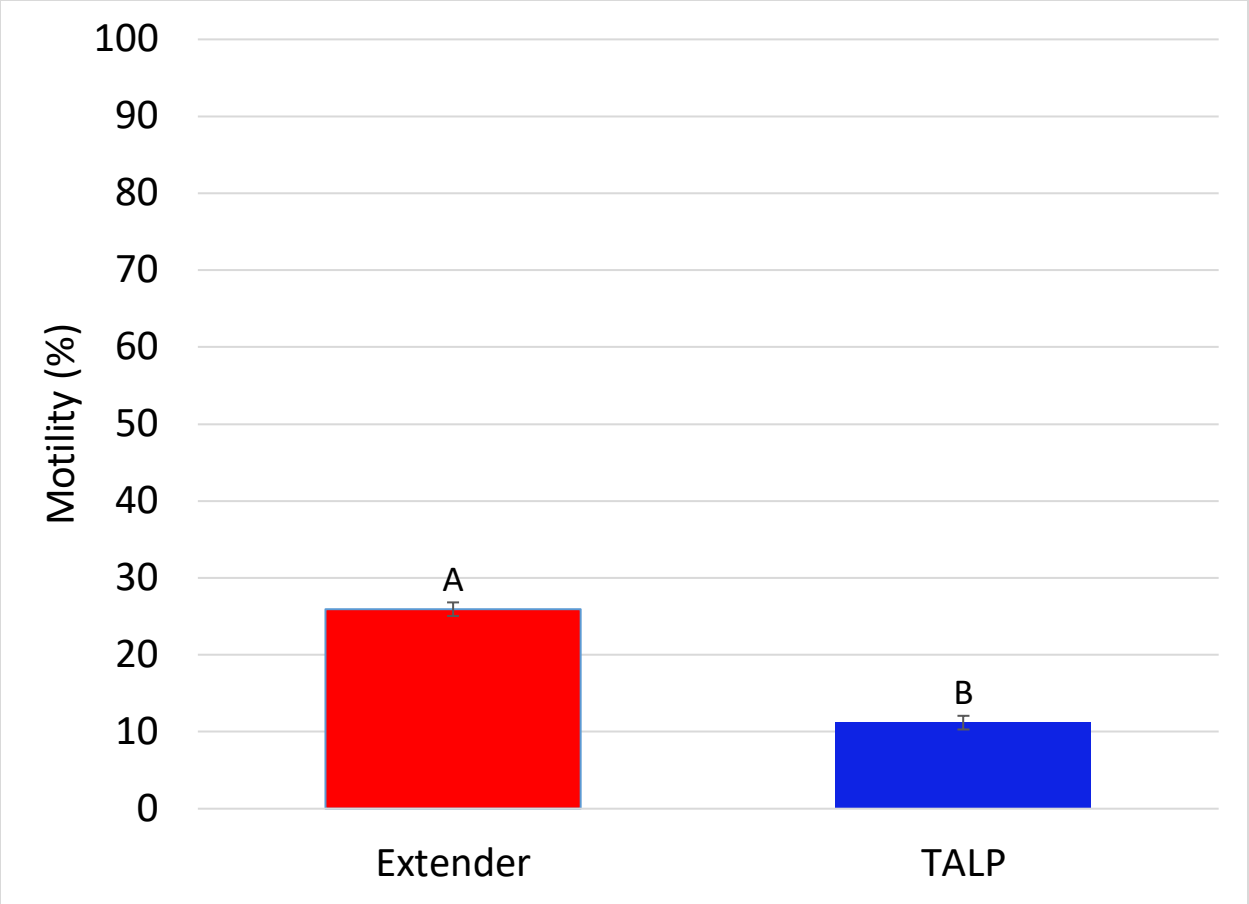

Figure 3.11: Effect of medium on sperm motility. Means averaged over time, temperature, and with and without glycans are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

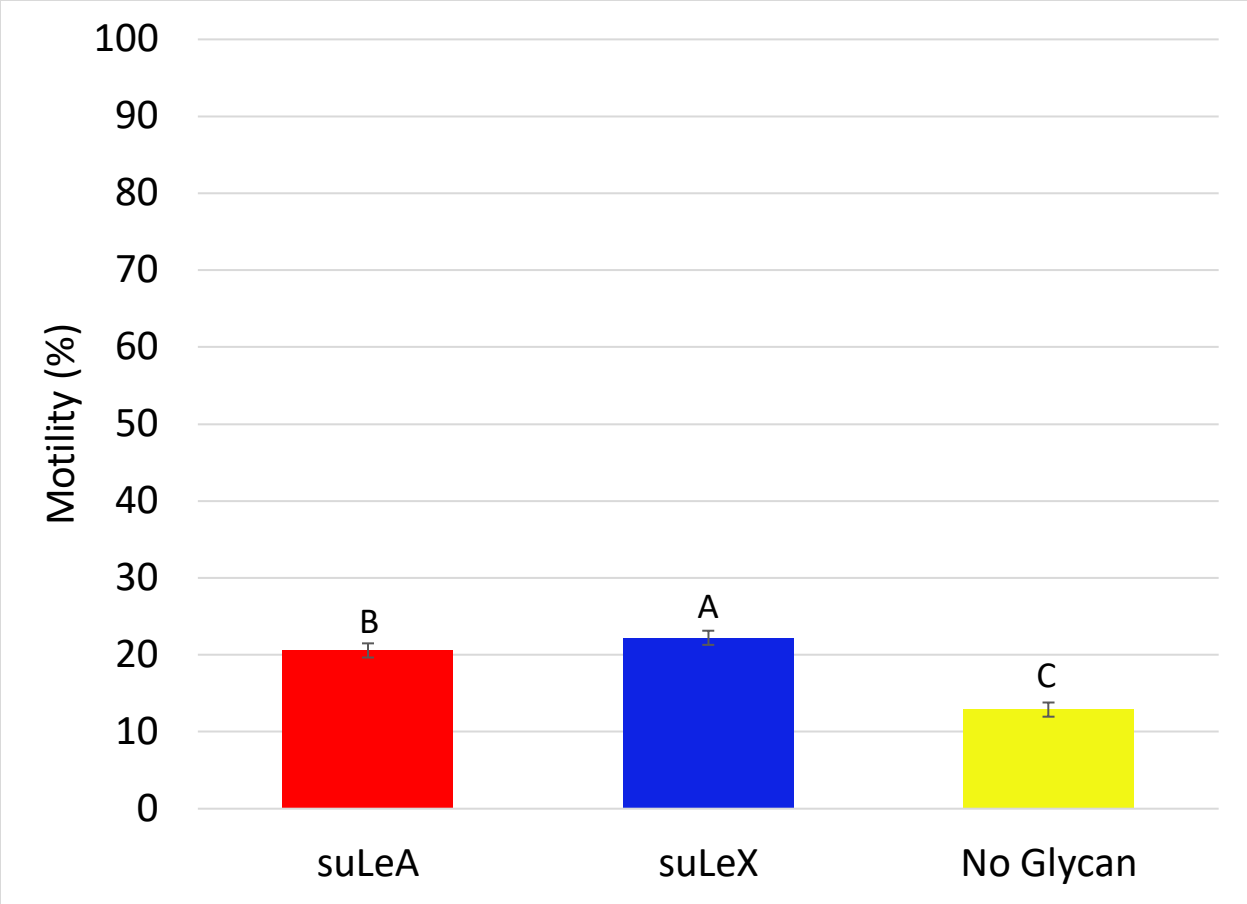

Figure 3.12: Effects of glycans on sperm motility. Means averaged over time, temperature and medium are shown. Means with different letter are significantly different ($p \leq 0.05$). The glycans used were $suLe^A$ (red), $suLe^X$ (blue), and no glycan (yellow).

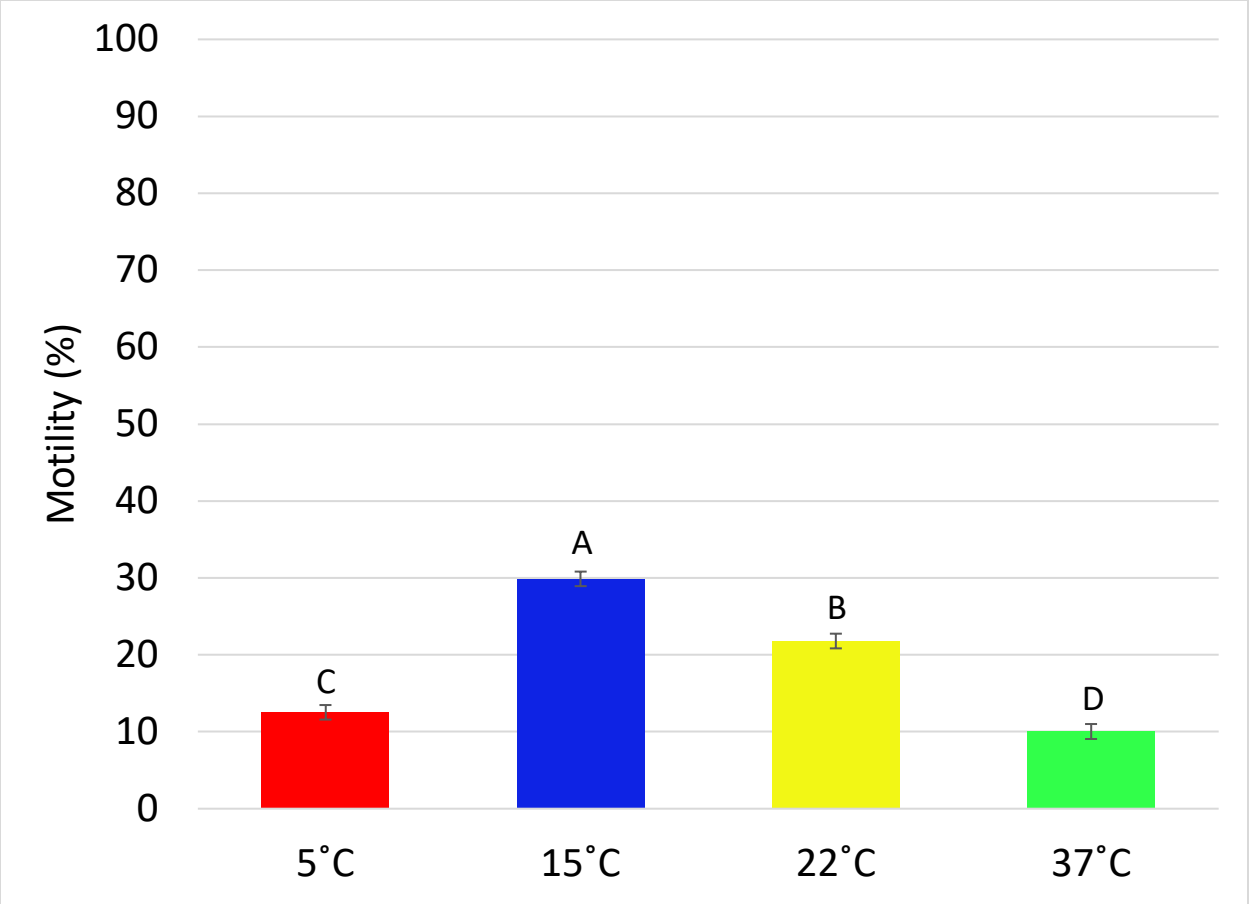

Figure 3.13: Effects of temperature on sperm motility. Means averaged over time, with and without glycans, and media are shown. Means with different letters are significantly different ($p \leq 0.05$). The temperatures used were 5°C (red), 15°C (blue), 22°C (yellow), and 37°C (green).

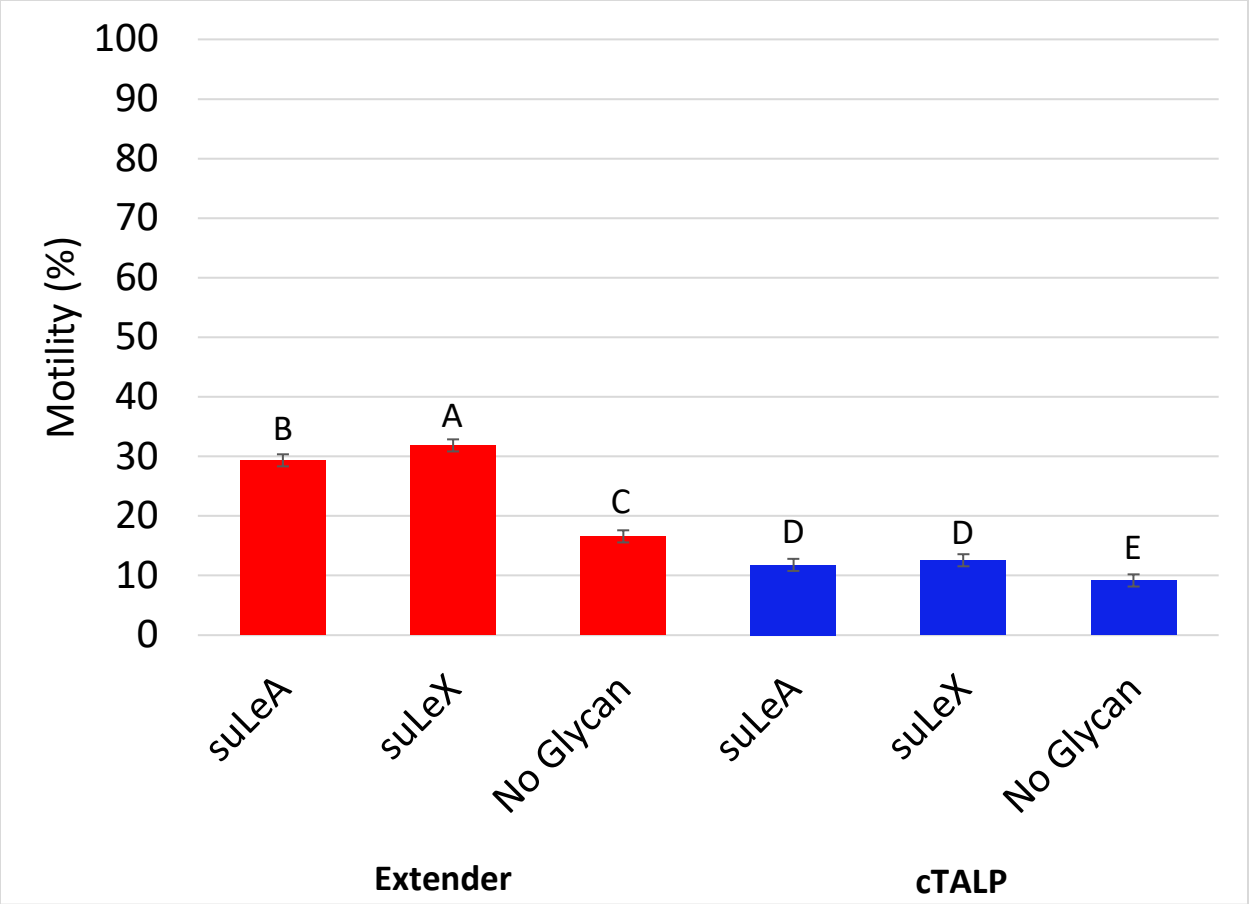

Figure 3.14: Effects of media and glycan on sperm motility. Means averaged over time and temperature are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

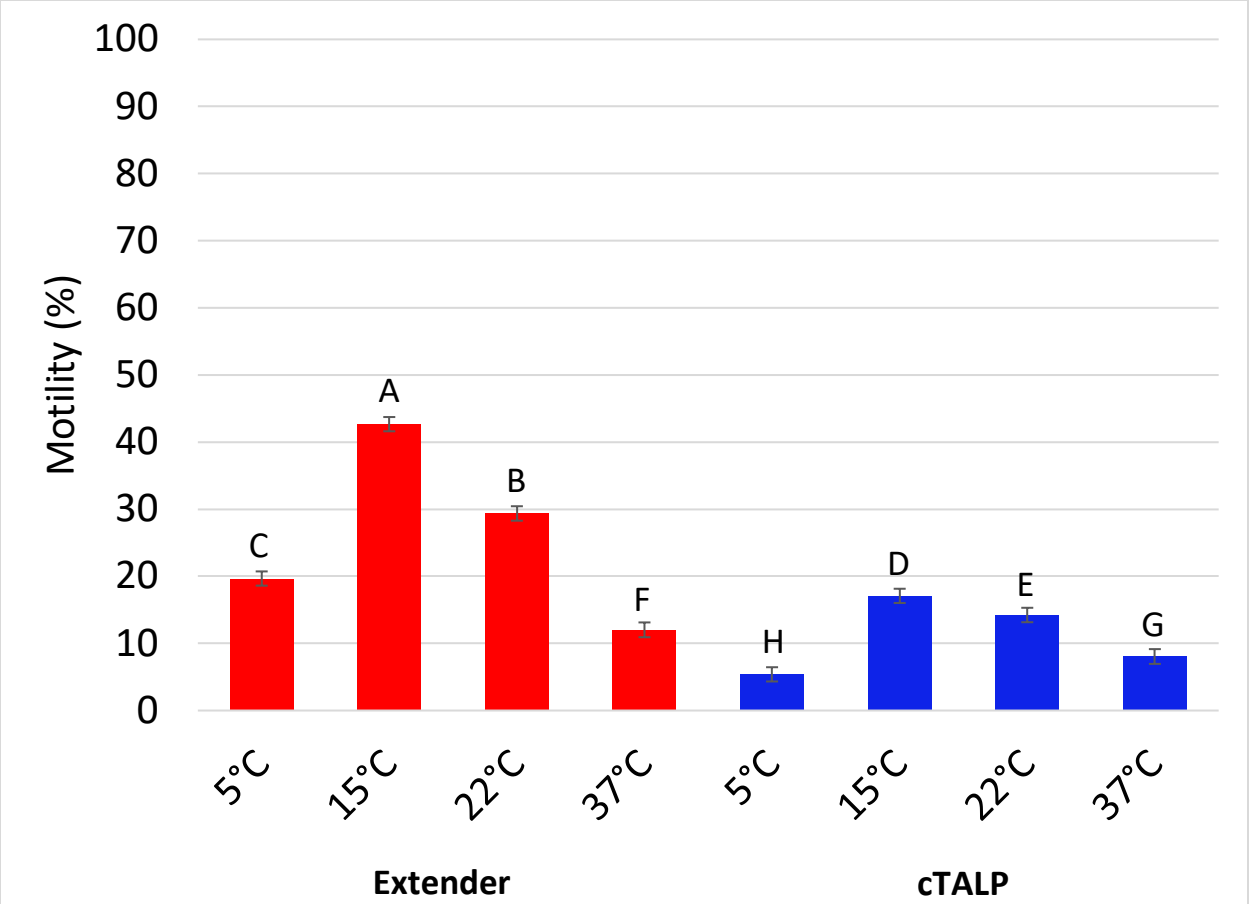

Figure 3.15: Effects of media and temperature on sperm motility. Means averaged over time and with and without glycans are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

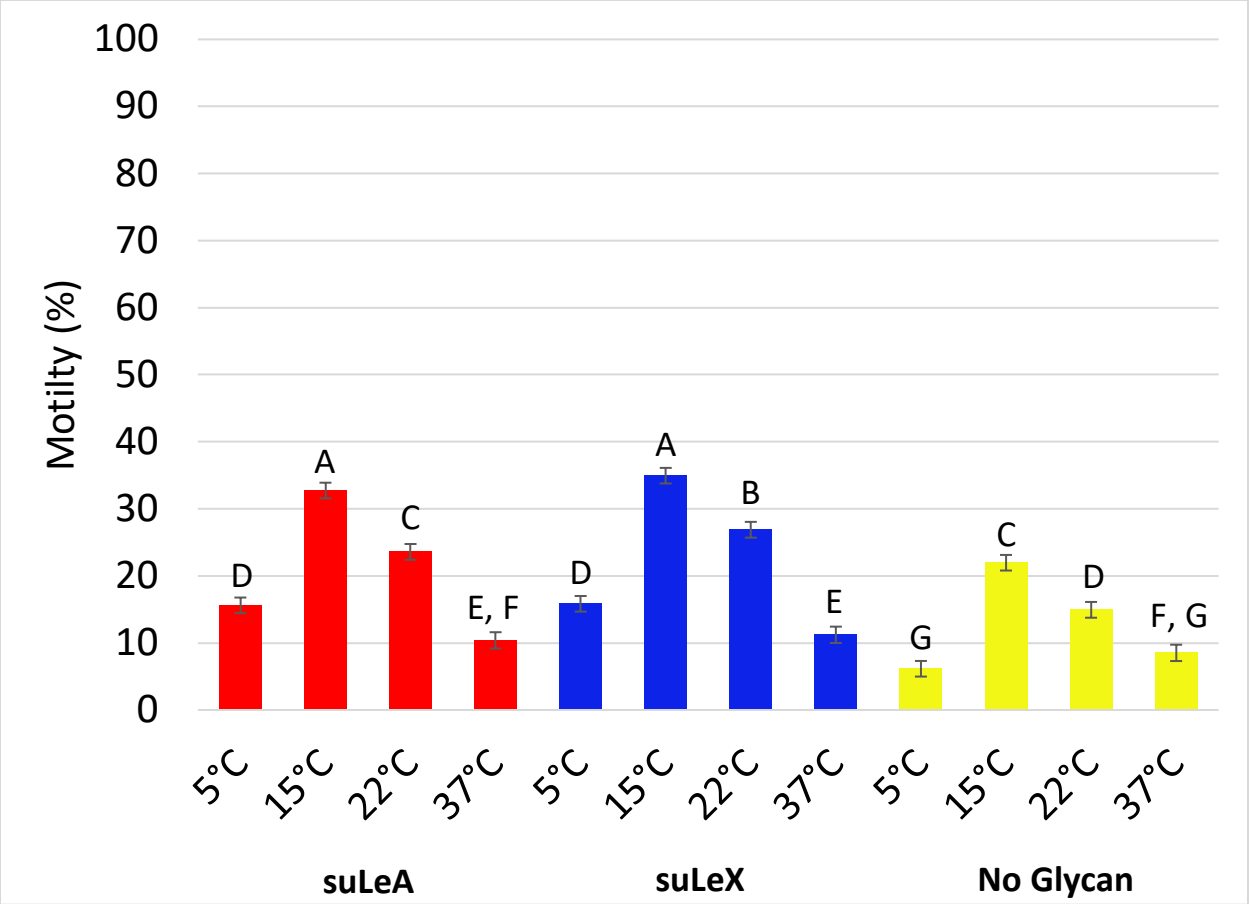

Figure 3.16: Effects of temperature and glycan on sperm motility. Means averaged over time and medium are shown. Means with different letter are significantly different ($p \leq 0.05$). The glycans used were $suLe^A$ (red), $suLe^X$ (blue), and no glycan (yellow).

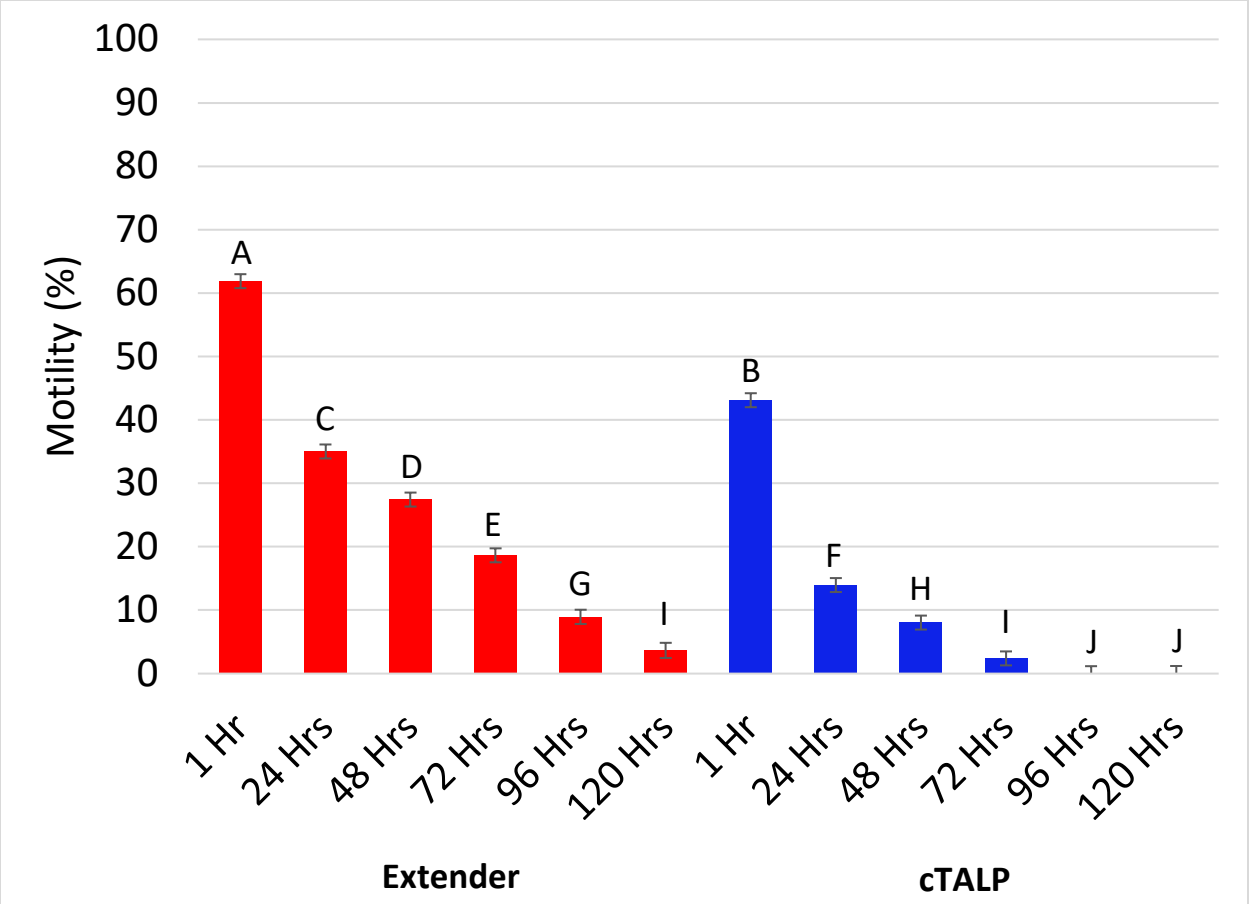

Figure 3.17: Effects of media and time on sperm motility. Means averaged over temperature and with and without glycans are shown. Means with different letters are significantly different ($p \leq 0.05$). The media used were FSRD4+ extender (red) and dmTALPC (blue).

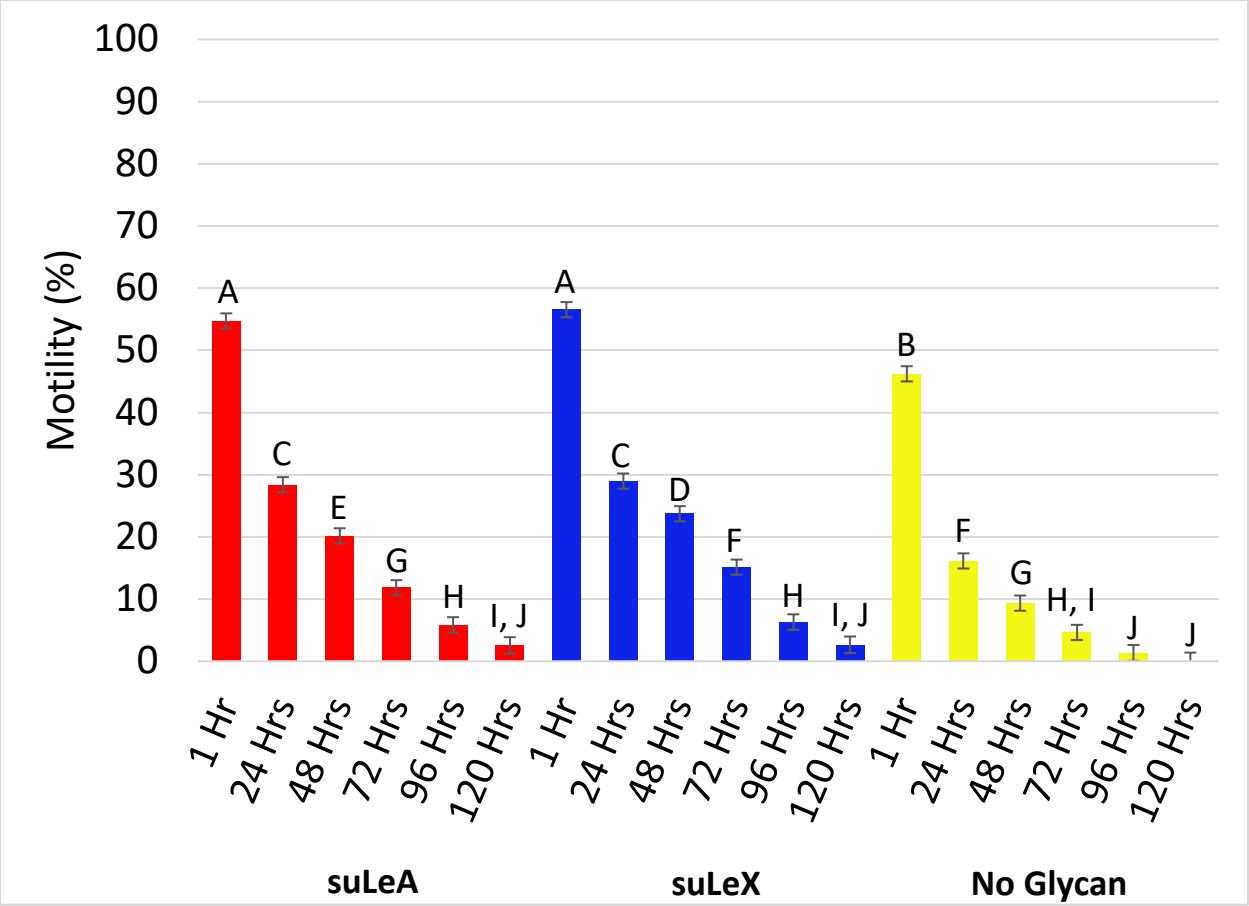

Figure 3.18: Effects of glycan and time on sperm viability. Means averaged over temperature and medium are shown. Means with different letter are significantly different ($p \leq 0.05$). The glycans used were $suLe^A$ (red), $suLe^X$ (blue), and no glycan (yellow).

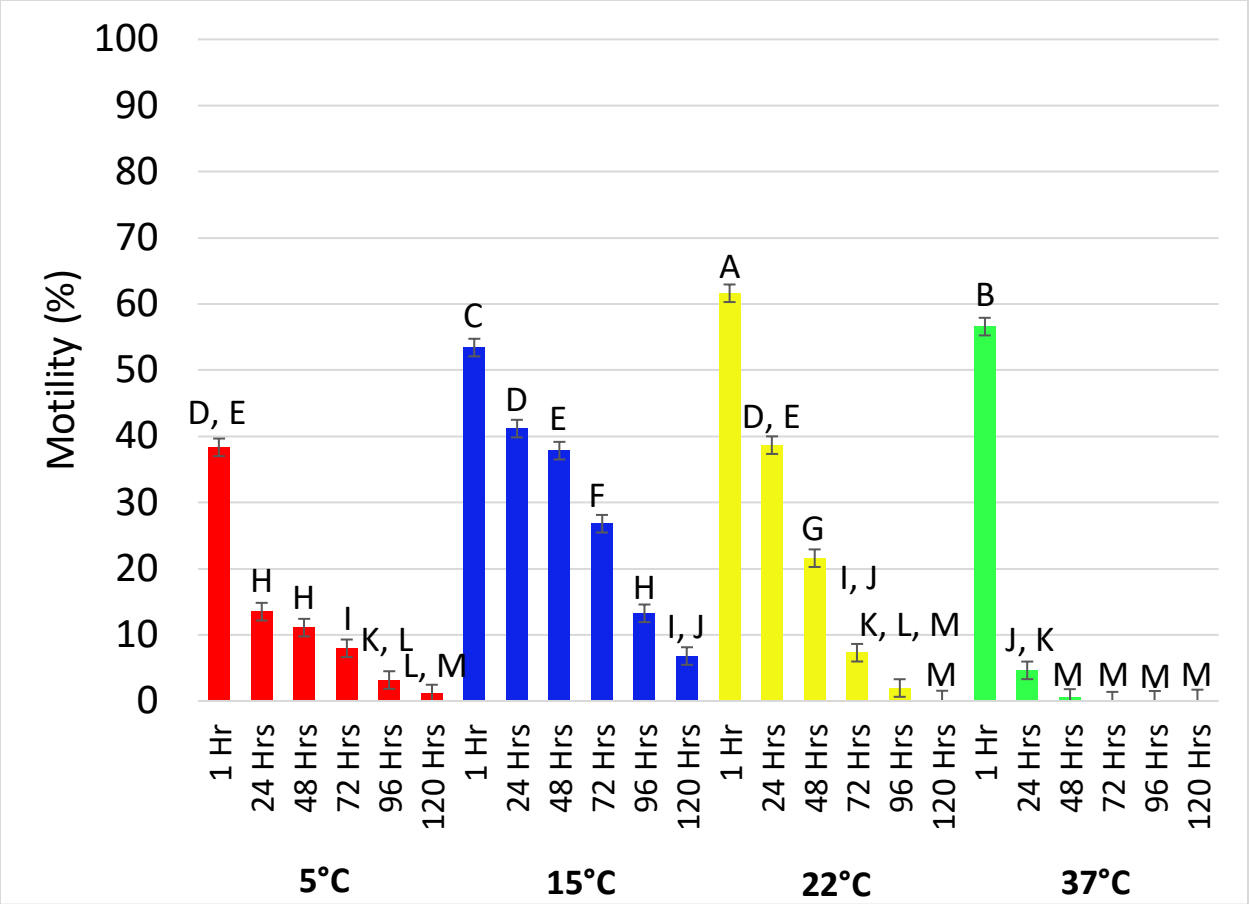

Figure 3.19: Effects of temperature and time on sperm motility. Means averaged over with and without glycans and medium are shown. Means with different letters are significantly different ($p \leq 0.05$). The temperatures used were 5°C (red), 15°C (blue), 22°C (yellow), and 37°C (green).

CHAPTER 4 – FUTURE DIRECTIONS AND OVERALL CONCLUSIONS

Summary

In many countries, the cattle industry heavily relies upon AI as a primary method of impregnation. Likewise, fresh vs frozen-thawed semen is another common divide where some countries such as Ireland and New Zealand breed cows almost exclusively with fresh semen, whereas other countries such as the United States rely more on frozen-thawed semen. The objectives were to verify if oviduct glycans, particularly $suLe^A$, improve the overall viability and motility of sperm compared to the non-glycan treatment, and to substantiate the effect of FSRD4+ extender on sperm viability and motility. Based on the objectives outlined and the results analyzed, the FSRD4+ extender significantly prolongs sperm viability compared to dmTALPC at 5°C, 15°C, and 22°C; the extender also significantly enhances the viability by 10-20% over a four-day timespan compared to dmTALPC. The $suLe^A$ glycan significantly improved the viability of bull sperm compared to the no-glycan treatment, but performed similarly to $suLe^X$. This is especially true for the combination of FSRD4+ with either glycan compared to any glycan treatment in dmTALPC and no-glycan treatment in extender. Sperm viability was not different between samples with $suLe^A$ and $suLe^X$ at 15°C and 22°C, but sperm with $suLe^A$ had significantly higher viability at both temperatures compared to the no-glycan treatment. In contrast, sperm viability with $suLe^X$ was not different than viability without added glycans at 15°C. Sperm motility followed a similar pattern. Sperm incubated with $suLe^A$ or $suLe^X$ performed similarly and were mostly significantly better than the no-glycan treatment at every time point except at 96 Hr. The extender had significantly higher motility at each time point from 1 hr to 120 hr compared to dmTALPC. Motility was best preserved in extender with $suLe^X$ over

suLe^{A}. Both glycans were better than the no-glycan control under these conditions. Similarly, both glycans maintained the highest and most significant motility at 15°C.

In short, highest sperm viability was obtained at 15°C, with either suLe^{A} or suLe^{X}, and FSRD4+ extender. Highest motility was also obtained at 15°C, with suLe^{A} or suLe^{X}, and FSRD4+ extender. Both oviduct glycans significantly improved the motility and viability of bovine sperm, especially for long-term storage. Likewise, sperm in FSRD4+ extender have significantly higher viability and motility compared to dmTALPC.

Future Directions

The basis of this project was an imperative outlook into the effects of soluble glycans on sperm lifespan. Aside from testing the efficacy of soluble glycans on sperm viability and motility, further trials need to be performed to understand how soluble glycans assist sperm. Previously, suLeA was shown to bind sperm and mediate sperm attachment to the oviduct. The effects of insoluble glycans, attached to oviduct cells or to beads, were studied, but research was limited to how bound glycans interact and affect sperm. However, this study is one of the first to explore the overall effect on sperm qualities with soluble glycans and sets an excellent foundation for deeper work with bovine sperm and follow-up work in other species.

In terms of more in-depth studies, measuring more sperm parameters would help identify the extent to which both the glycans and the FSRD4+ extender prolong sperm longevity. Further trials should be conducted to better understand how sperm are affected by oviduct glycans, reactive oxygen species production, and acrosome reaction frequency. Unfortunately, neither motility nor morphology alone is able to accurately predict sperm fertility (Gadea et al., 2004). Oviduct glycans and the extender may also reduce the number of degenerative acrosome

reactions, which can be observed by staining the acrosome with Coomassie Blue G250 or FITC-PSA (fluorescein isothiocyanate-labelled Pisum sativum agglutinin). Both of these techniques can distinguish between intact and damaged acrosomes. Aside from identifying physical characteristics, sperm can also be damaged by reactive oxygen species (ROS). The sperm reservoir selects sperm that have normal DNA or minimal chromatin damage (Fazeli et al., 2004). With exposure to ROS, sperm membrane bilayer and DNA/chromatin integrity can be damaged through oxidation. ROS production can be detected by fluorescent probes. As for DNA/chromatin damage, Sperm Chromatin Structure Assay (SCSA), terminal deoxynucleotidyl transferase dUTP Nick-End Labeling assay (TUNEL), and Comet assay are techniques that can determine DNA damage by measuring DNA strand breaks. Although independently these assays show some relationship to fertility, the combination of all of them can help determine how FSRD4+ extender and glycans prolong lifespan.

Other factors to consider when determining the usefulness of these results in practical situations is whether it will have an impact on fertility. The first possible dilemma is sperm were bound to soluble glycans could have difficulties rebinding the epithelial cells in the oviduct. Pre-binding could cause interference with future binding. However, soluble glycans do exist in the uterus and cervix. Experiments of sperm bound to soluble glycans via *in vitro* and *in vivo* experiments would be helpful in determining if any binding hindrances or fertility declines occur. To continue, testing the binding strength between soluble and insoluble glycans would be helpful in determining if sperm bind more readily with the epithelium or not. This also raises the question of possible competition existing in the oviduct between highly selective epithelial cells and soluble glycans.

One limitation of this project was semen being shipped overnight chilled and already one day-old post-collection semen at the time of processing. Future trials could be set up to use fresh semen to see how the extender and glycans affects sperm based on the aforementioned parameters. A critical issue that constantly interfered was fungal and bacterial contamination, even with the addition of an antibacterial/antimycotic solution. My preliminary experiments indicated that contamination was coming from the semen itself. If contamination could be reduced, longer trials could be performed testing effects on viability of fresher sperm. Lastly, it would be helpful to understand how well FSRD4+ extender competes with other extenders designed for liquid storage such as Caprogen, BIOXcell, and CEP-2.

As mentioned before, further experiments should be tested with other species' semen, specifically with porcine semen. A sub-focus of this project was to see if liquid-stored semen could contend with frozen-thawed semen, but in bovine. Since use of liquid-stored semen and AI in the US is ubiquitous in the porcine industry, it would be beneficial to determine if soluble glycans prolong porcine sperm fertility. On the topic of frozen-thawed semen, trials comparing effects of oviduct glycans on fresh and frozen-thawed sperm might reveal if oviduct glycans could extend the lifespan of frozen-thawed sperm after AI. It is also possible to add oviduct glycans to sperm before deep-freezing to see if it affects the acrosome status or maintains or mitigates the issues of decreased motility and viability for sperm surviving the thawing process. Cryo-capacitation negatively affects sperm, which leads to reduced fertility due to capacitation-like changes in thawed sperm (Thomas et al., 2006; Talukdar et al., 2015). Because of these changes, cryopreserved sperm have reduced survivability and longevity in the female reproductive tract (Talukdar et al., 2015). Glycans may slow the capacitation-inducing effects of sperm cryopreservation and, thereby, extend sperm lifespan.

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APPENDIX A – PROTOCOL FOR GLYCAN-BOUND BEADS BULL SPERM VIABILITY

The thesis had originally been focusing on the effects of glycan-bound beads on the viability of bull sperm. Temperature and media were other factors that were being measured aside from $suLe^A$ and $suLe^X$. After months of troubleshooting primarily concentrating on poor sperm-bead binding and aggressive fungal contamination, the thesis was revised to soluble glycans instead of bead-bound glycans. The reasoning for this alteration was because we believed soluble glycans would produce the same or similar physiological effects on bull sperm as compared to the bead-bound glycans.

Preparation of Glycan-Bound Beads

1. Pipette 20 µL of Streptavidin Sepharose® High Performance from Sigma-Aldrich (Saint Louis, Missouri) into a 1.5 mL microcentrifuge tube.
2. Add 1.0 mL of 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) to the beads and thoroughly mix the beads.
3. Centrifuge the beads at 184xg for 2 min.
4. Discard the supernatant and repeat this process twice for a total of 3 washes with 1X PBS.
5. After discarding the supernatant from the third wash, add 60 µL (1 mg/mL) of biotinylated glycan-PAA (glycan linked to biotinylated 30 kDa chain of polyacrylamide) to the beads in the tube.
6. Incubate the beads at room temperature for 2 hr, with intermittent tube flicking every 5 min throughout the incubation period.

7. Wash the beads with 1 mL of dmTALP (non-capacitating) and centrifuge at 1500 rpm for 2 minutes.
8. Discard the supernatant and repeat the previous step for a total of 2 washes with dmTALP.
9. Resuspend the biotinylated beads with 100 µL of dmTALP and store the beads at 4°C.

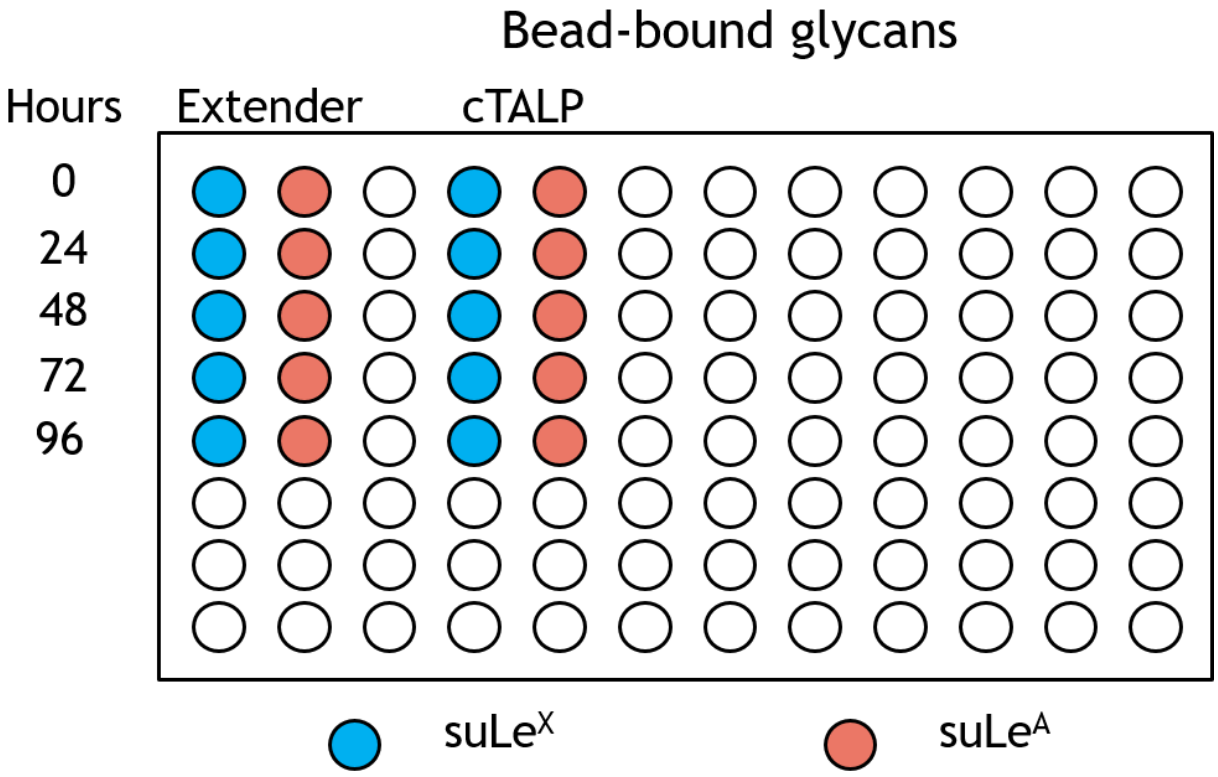

Figure A.1: An aerial view of a 96-well plate with the bead-bound glycans.

Plate Preparation

1. Label 4 96-well plates; each plate represents a specific temperature (5°C, 15°C, 22°C, and 37°C).
2. Label the columns of each plate with each media/glycan combination (suLe^X/extender, suLe^A/extender, suLe^X/dmTALPC, suLe^A/dmTALPC).

3. Label the rows of each plate with hr counts from 0-96 hr every 24 hr, i.e., 0, 24, 48, 72, and 96 hr.
4. In each of the wells labeled with a specific glycan, pipette in 2 μL of the respective glycan-bound beads.

Sperm Preparation

1. Prepare 5 15-mL conical tubes for each of the following contents: bull semen, Percoll wash, dmTALPC for the trials, dmTALPC for the sperm washes, and FSRD4+ extender.
2. Warm 10 mL of dmTALPC for the trials, 15 mL of dmTALPC for the washing, 10 mL of FSRD4+ extender, 4 mL of bull semen, and 10 mL of Percoll wash (5.4mL Percoll, 0.6mL 10X HBS (1.3 M NaCl, 40 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$), 4 mL dmTALPC) at 37°C for 15 minutes.
3. Pipette 3 mL of the bull semen onto the top of the Percoll wash; do not mix the Percoll and semen.
4. Centrifuge the tube at 796xg for 12 min.
5. Discard the supernatant and resuspend the pellet with 5 mL of warmed dmTALPC.
6. Centrifuge the tube at 195xg for 5 min.
7. Discard the supernatant and resuspend the pellet with 5 mL of warmed dmTALPC.
8. Centrifuge the tube at 195xg for 5 min for a second time.
9. Discard the supernatant and resuspend the pellet with 5 mL of warmed dmTALPC.
10. Using the hemocytometer, determine the sperm count and dilute the washed sperm down to a concentration of 2.0×10^6 sperm/mL in both the dmTALPC and FSRD4+ extender conical tubes.

11. Add 200 μL of the diluted sperm to each labeled wells for all four of the 96-well plates.
12. Place one 96-well plate in each of their respective temperatures (5°C, 15°C, 22°C, and 37°C) in the dark to prevent light from inactivating the bactericide/fungicide
13. After the incubation period is over, prepare the sperm for viability counts and count using a Zeiss Axiovert 25 (Zeiss Microscopy, LLC, Thornwood, NY) at 200X magnification.

Determining Sperm Concentration by Hemocytometer

1. Make a 1:100 dilution of sperm:water in a 1.5 mL microcentrifuge tube.
2. Place 10 μL of the dilution in the top and bottom grooves of the hemocytometer.
3. Wait 2 min before counting for the sperm to settle.
4. Tally the top and bottom sections separately by counting the 5 large squares where the horizontal and vertical lines meet. 4 of the 5 squares are at the vertices in each section and the fifth square is in the center. Each square contains 16 smaller squares; count the total sperm that are within the 5 large squares.
5. To determine the sperm concentration, average the total sperm count of the top and bottom sections of the hemocytometer, multiply by the dilution factor, and 50,000 if you count the 5 large squares or 10,000 if you count all 25 large squares.
    a. Layout: (Total sperm count/2) x 100 x 50,000 = X sperm/mL
6. After the total sperm concentration is calculated, dilute the sperm down to 2.0×10^6 sperm/mL.

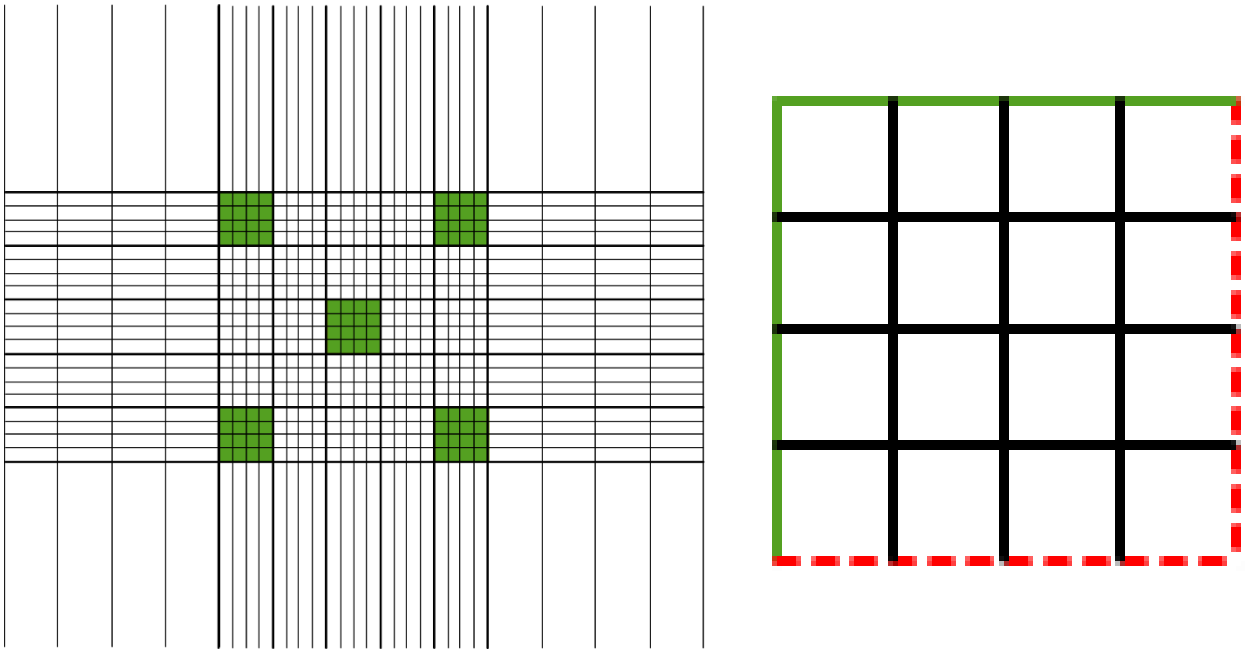

Figure A.2: The left image is of a whole view of the hemocytometer. Horizontal and vertical lines intersect to create squares that help in counting sperm and calculating the sperm concentration. The boxes highlighted green are the 5 large squares to be counted. Count all of the sperm inside the 5 large boxes. If sperm are resting on the thicker borders of the squares only count sperm on the western and northern borders of each large square, which is shown in the right image in the green, solid lines.

APPENDIX B – SPERM/GLYCAN-BEAD BINDING ADDITIONAL INFORMATION

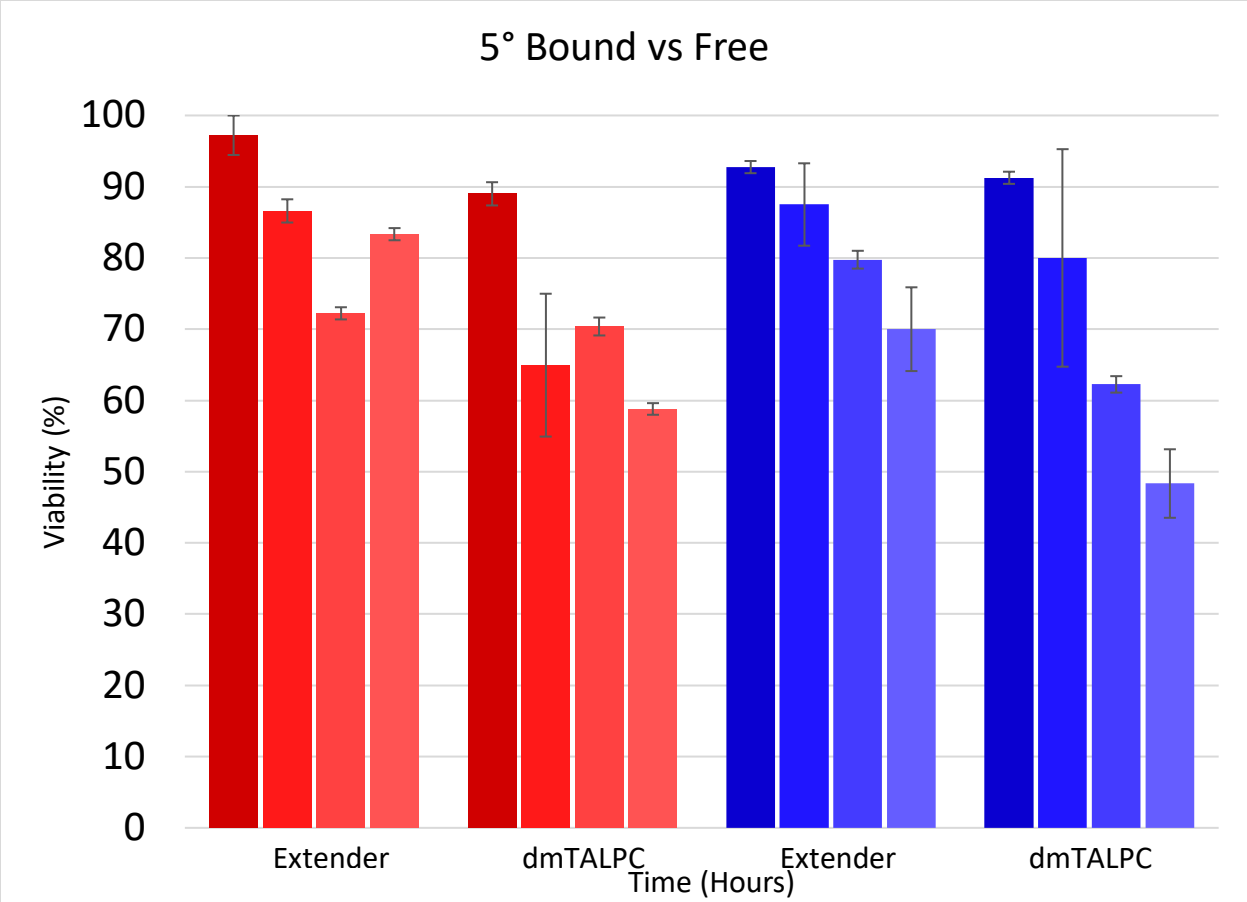

Figure B.1: Viability of Bull Sperm at 5°C. Graph representing the viability of pooled bull semen from 4 treatments at 4 time points at 5°C. From left to right, the treatments are FSRD4+ extender with suLe^{A}-bound beads, dmTALPC with suLe^{A}-bound beads, FSRD4+ extender without suLe^{A}-bound beads, and dmTALPC without suLe^{A}-bound beads. Red represents bead-bound sperm, and blue represents free sperm. For each treatment, the leftmost column represents the 1 hr timepoint to 24 hr, 48 hr, and 72 hr as the rightmost column. No statistical analysis was performed due to varying number of replicates and low numbers of cells.

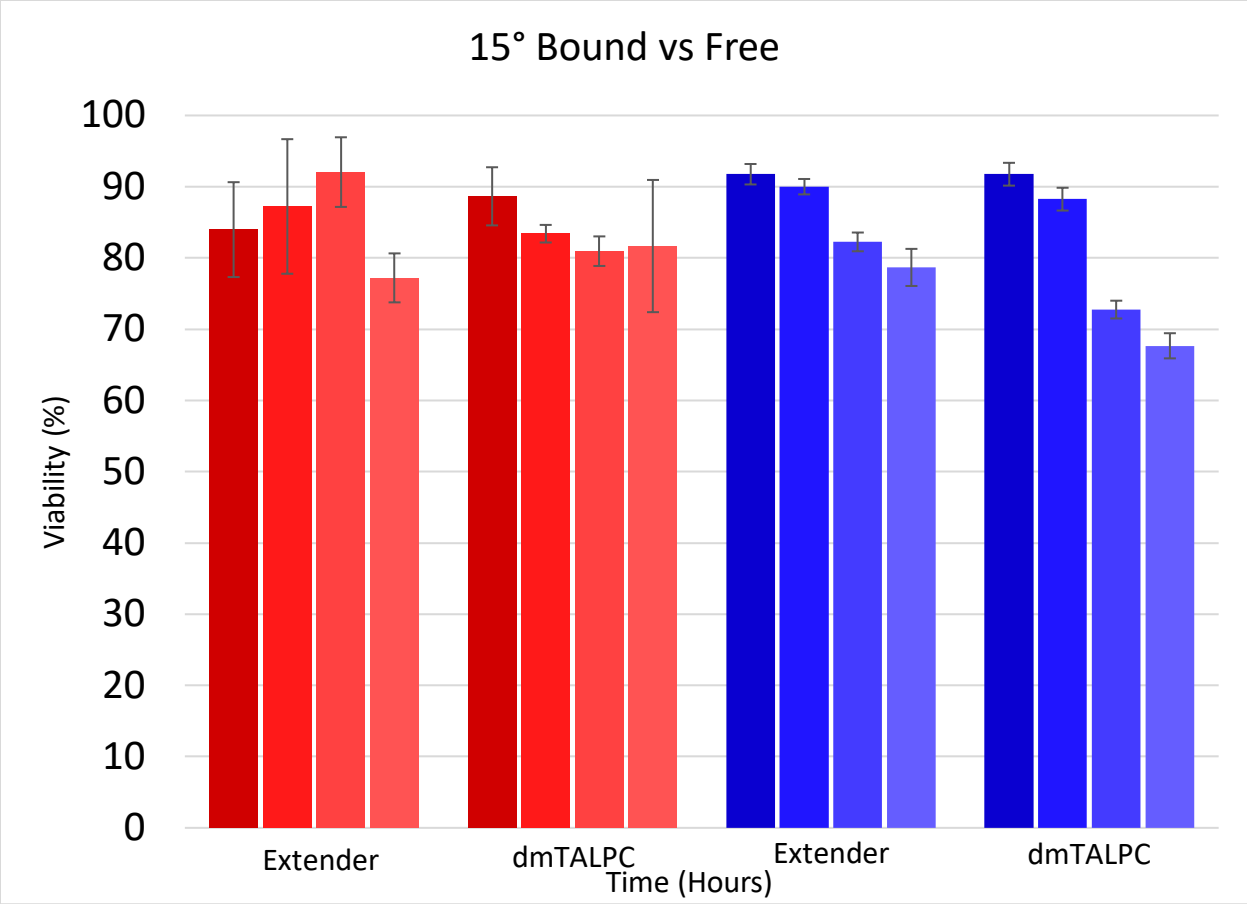

Figure B.2: Viability of Bull Sperm at 15°C. Graph representing the viability of pooled bull semen from 4 treatments at 4 time points at 15°C. From left to right, the treatments are FSRD4+ extender with suLe^{A}-bound beads, dmTALPC with suLe^{A}-bound beads, FSRD4+ extender without suLe^{A}-bound beads, and dmTALPC without suLe^{A}-bound beads. Red represents bead-bound sperm, and blue represents free sperm. For each treatment, the leftmost column represents the 1 hr timepoint to 24 hr, 48 hr, and 72 hr as the rightmost column. No statistical analyses were ran on the data due to varying number of replicates and low counts.

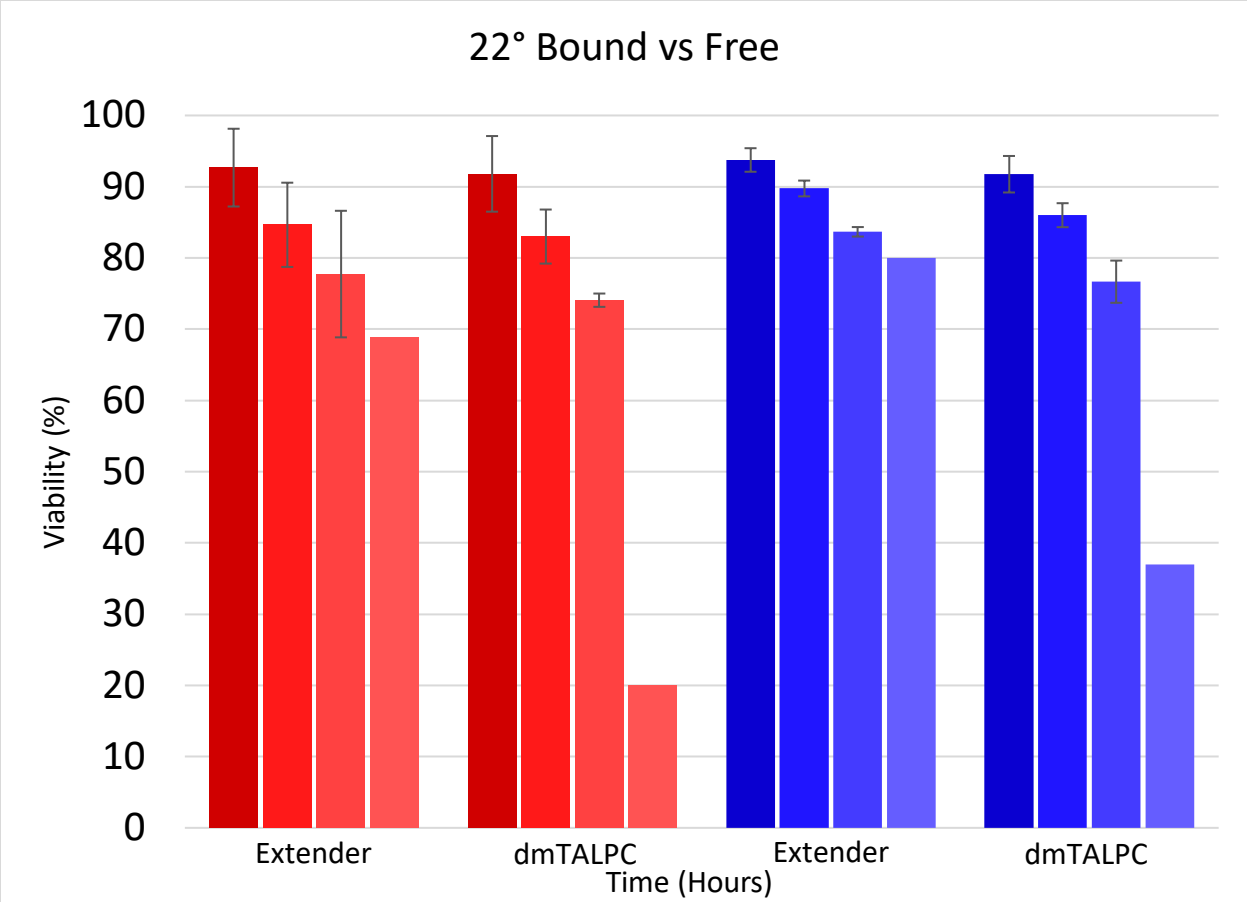

Figure B.3: Viability of Bull Sperm at 22°C. Graph representing the viability of pooled bull semen from 4 treatments at 4 time points at 22°C. From left to right, the treatments are FSRD4+ extender with suLe^{A}-bound beads, dmTALPC with suLe^{A}-bound beads, FSRD4+ extender without suLe^{A}-bound beads, and dmTALPC without suLe^{A}-bound beads. Red represents bead-bound sperm, and blue represents free sperm. For each treatment, the leftmost column represents the 1 hr timepoint to 24 hr, 48 hr, and 72 hr as the rightmost column. No statistical analysis was performed due to varying number of replicates and low numbers of cells.

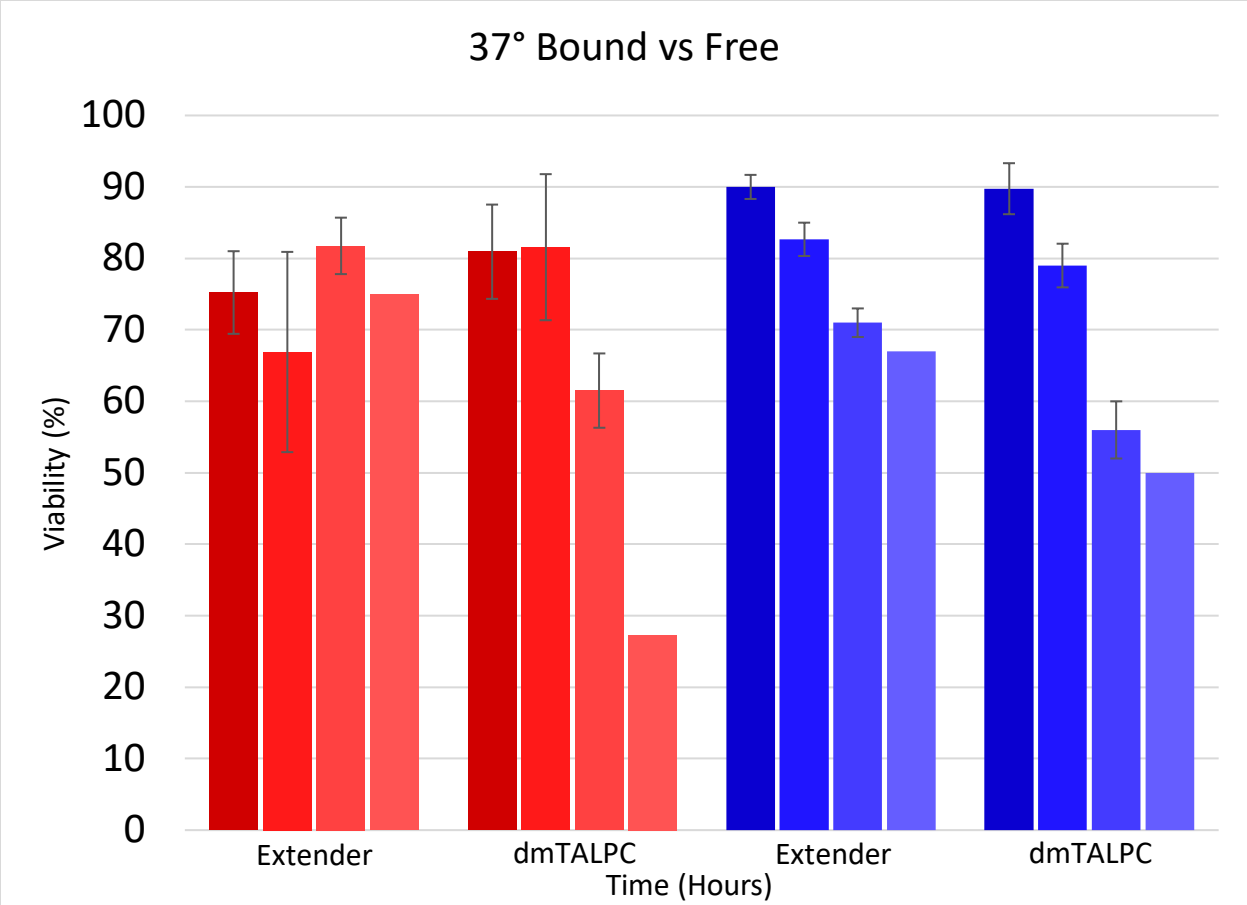

Figure B.4: Viability of Bull Sperm at 37°C. Graph representing the viability of pooled bull semen from 4 treatments at 4 time points at 37°C. From left to right, the treatments are FSRD4+ extender with suLe^{A}-bound beads, dmTALPC with suLe^{A}-bound beads, FSRD4+ extender without suLe^{A}-bound beads, and dmTALPC without suLe^{A}-bound beads. Red represents bead-bound sperm, and blue represents free sperm. For each treatment, the leftmost column represents the 1 hr timepoint to 24 hr, 48 hr, and 72 hr as the rightmost column. No statistical analysis was performed due to varying number of replicates and low numbers of cells.

APPENDIX C – VIABILITY AND MOTILITY GRAPHS

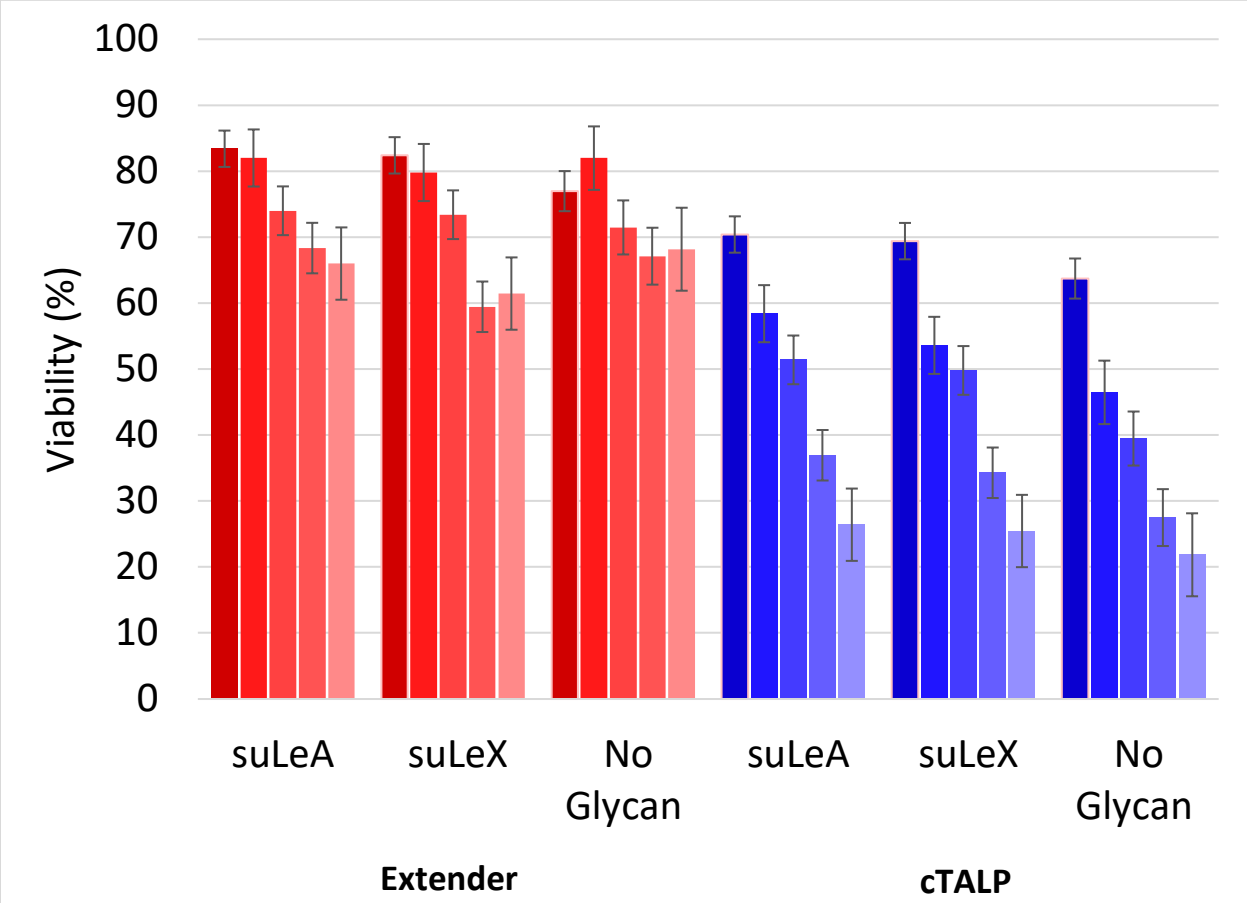

Figure C.1: Viability of Bull Sperm at 5°C over a 96 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 5 time points at 5°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 5 time points: 1 hr, 24 hr, 48 hr, 72 hr, and 96 hr.

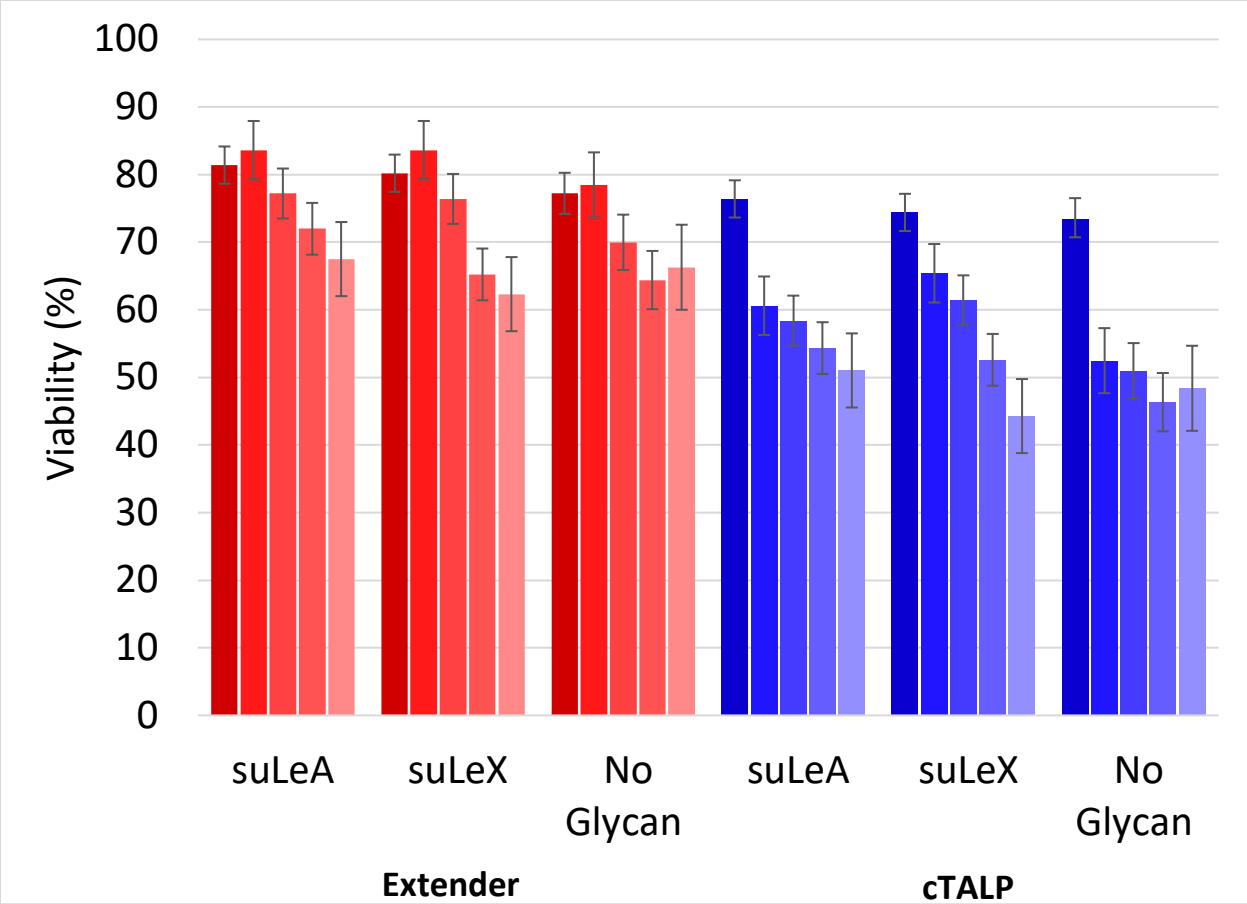

Figure C.2: Viability of Bull Sperm at 15°C over a 96 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 5 time points at 15°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 5 time points: 1 hr, 24 hr, 48 hr, 72 hr, and 96 hr.

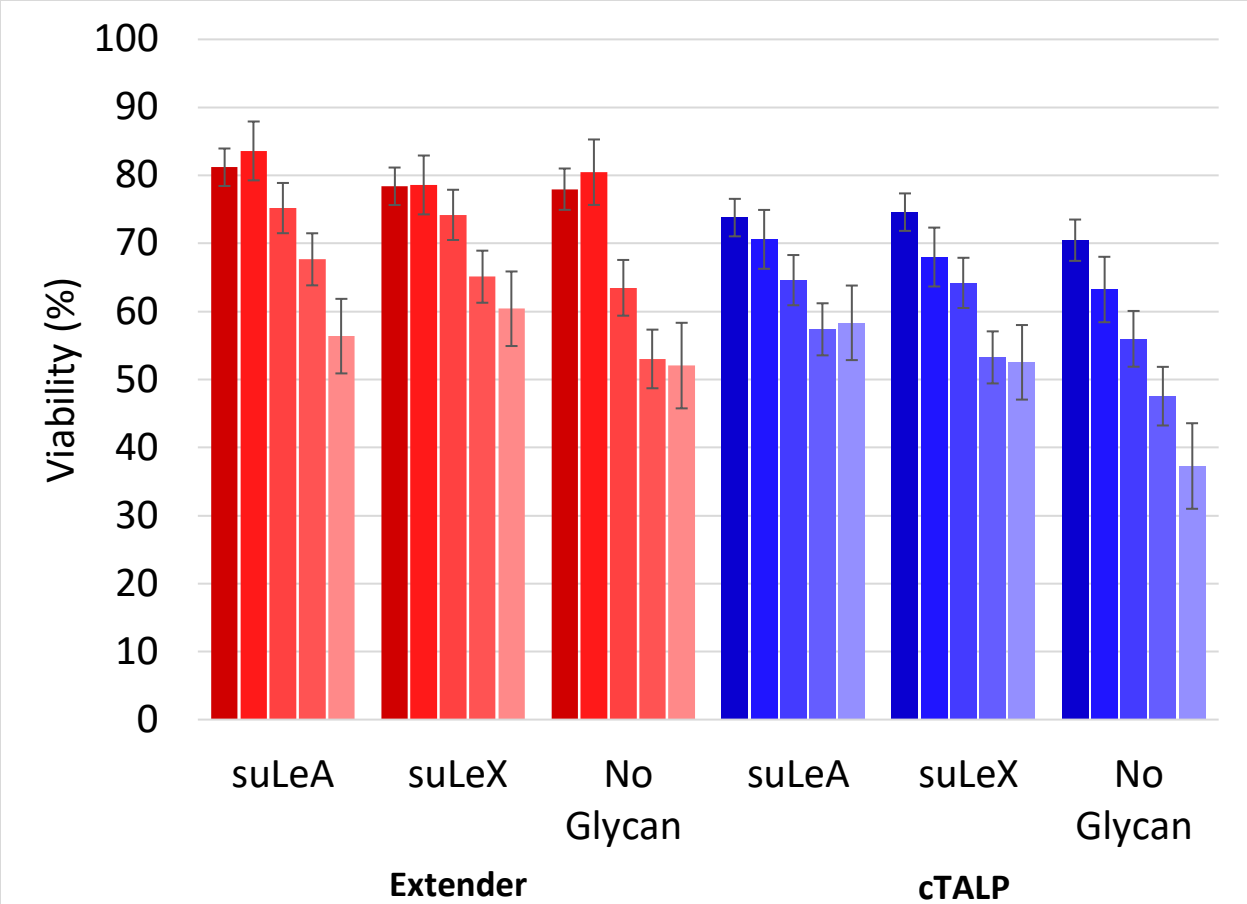

Figure C.3: Viability of Bull Sperm at 22°C over a 96 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 5 time points at 22°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 5 time points: 1 hr, 24 hr, 48 hr, 72 hr, and 96 hr.

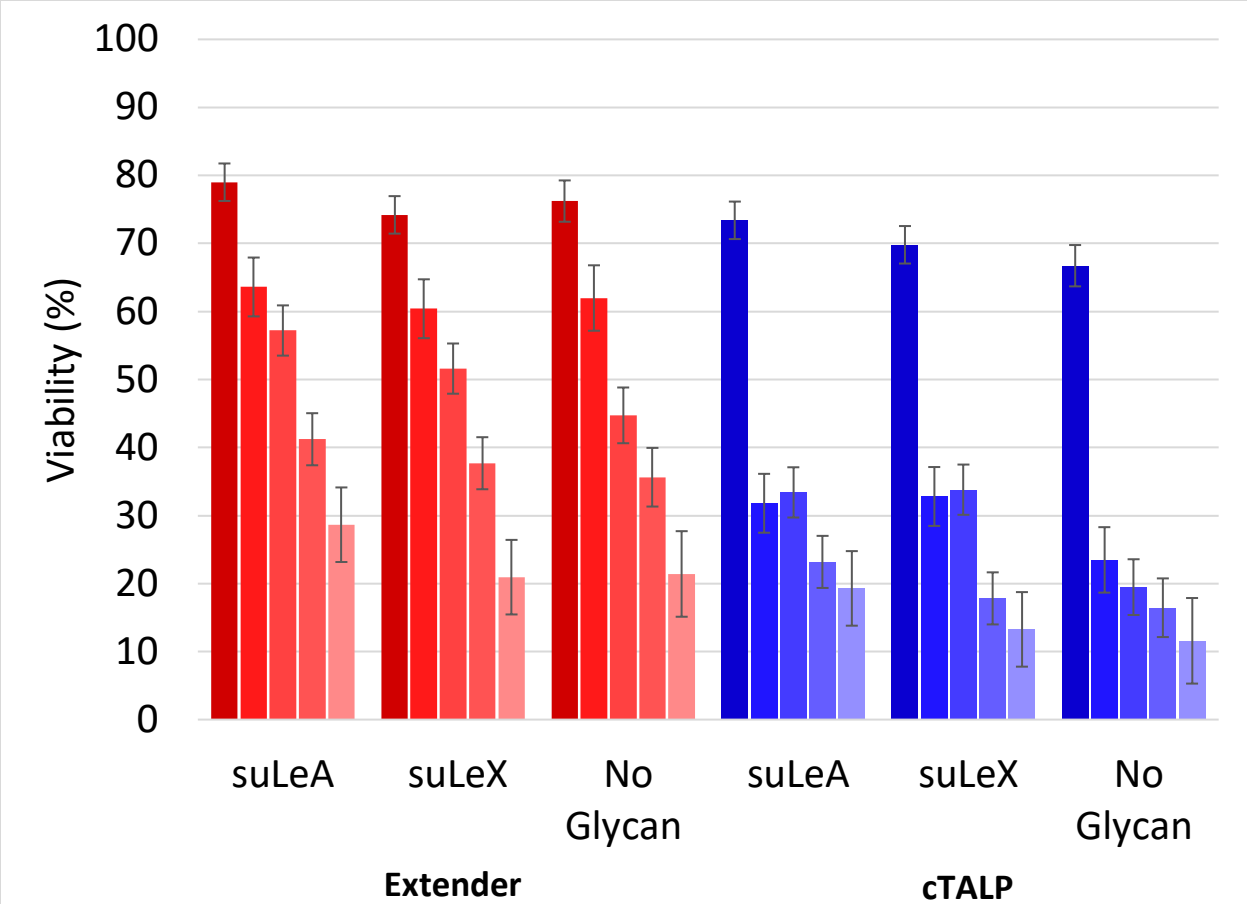

Figure C.4: Viability of Bull Sperm at 37°C over a 96 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 5 time points at 37°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 5 time points: 1 hr, 24 hr, 48 hr, 72 hr, and 96 hr.

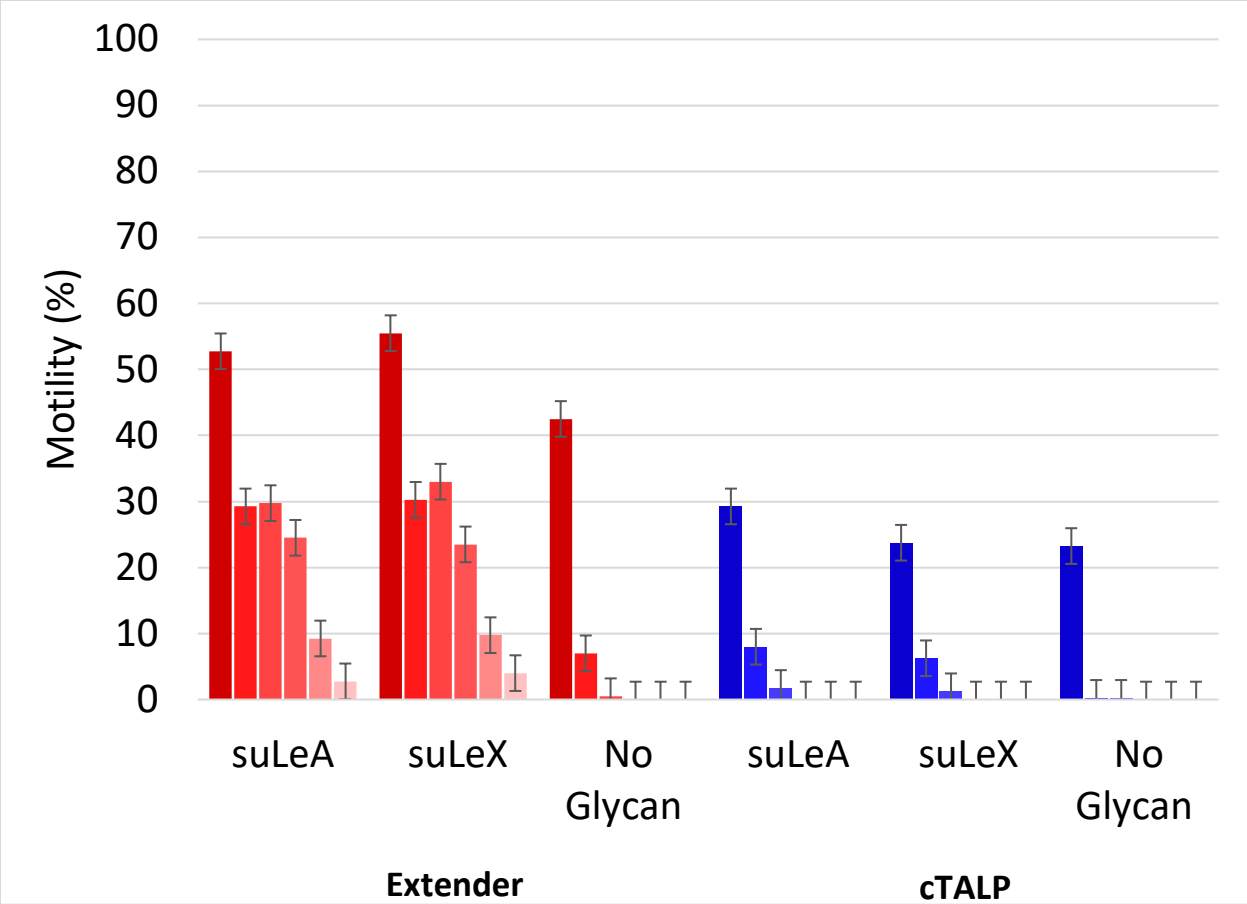

Figure C.5: Motility of Bull Sperm at 5°C over a 120 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 6 time points at 5°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 6 time points: 1 hr, 24 hr, 48 hr, 72 hr, 96 hr, and 120 hr.

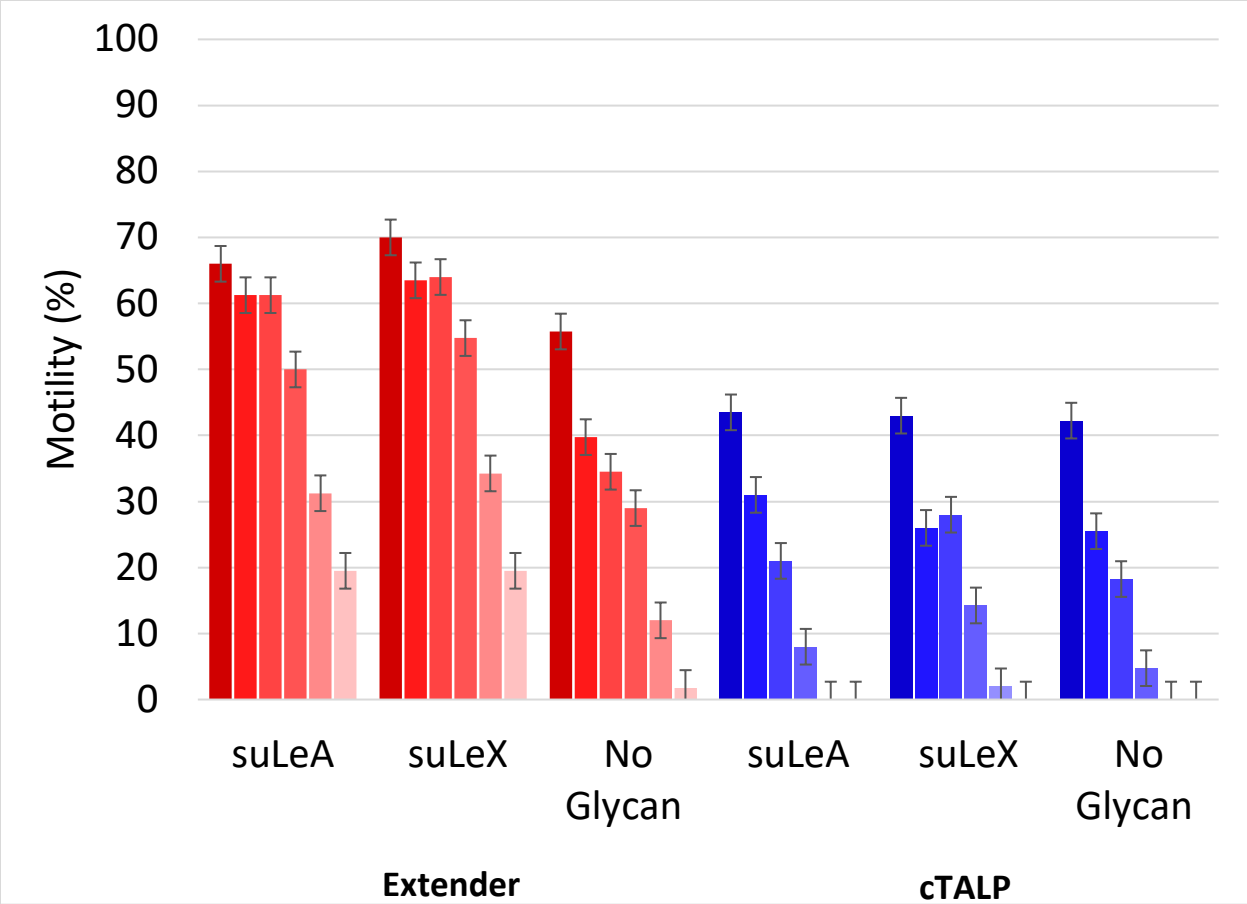

Figure C.6: Motility of Bull Sperm at 15°C over a 120 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 6 time points at 15°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 6 time points: 1 hr, 24 hr, 48 hr, 72 hr, 96 hr, and 120 hr.

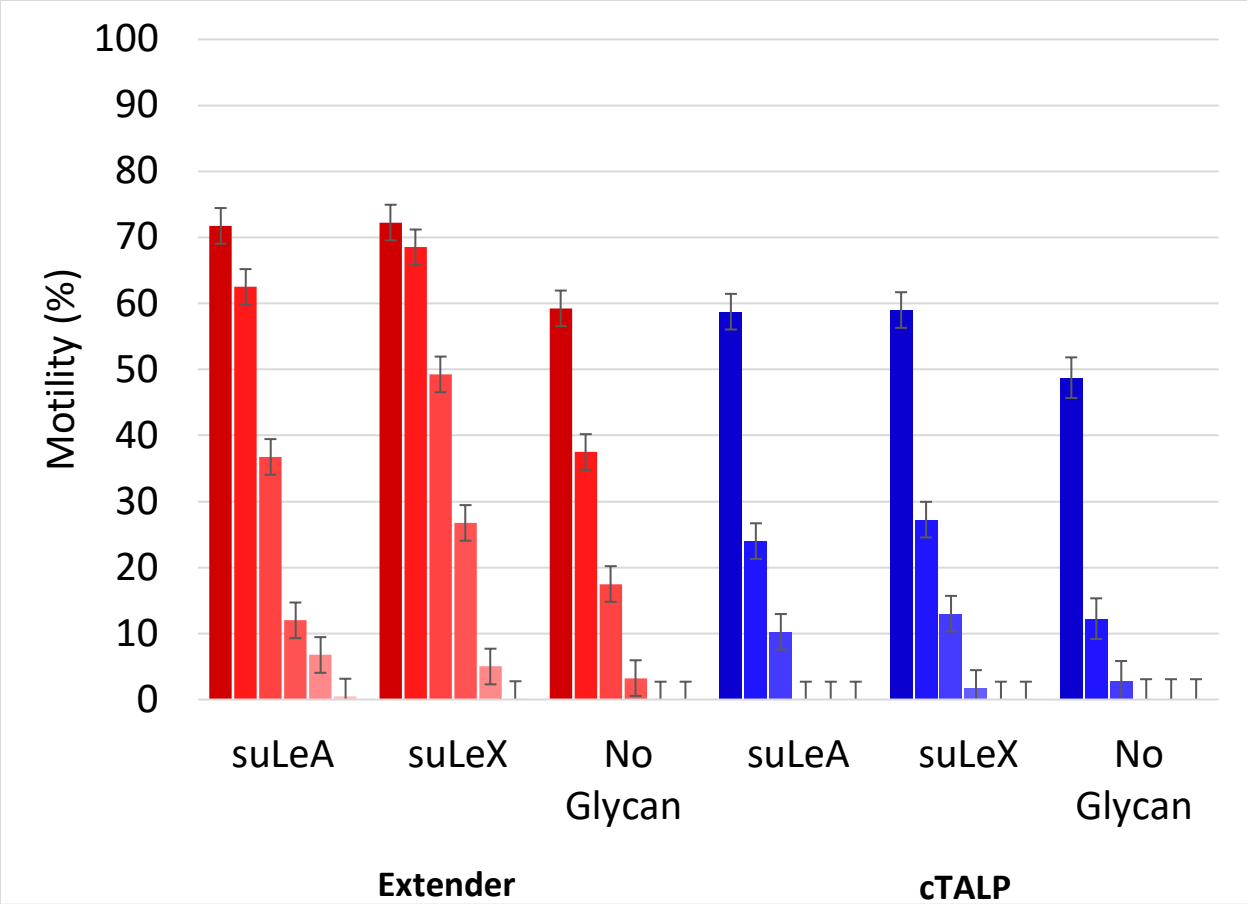

Figure C.7: Motility of Bull Sperm at 22°C over a 120 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 6 time points at 22°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 6 time points: 1 hr, 24 hr, 48 hr, 72 hr, 96 hr, and 120 hr.

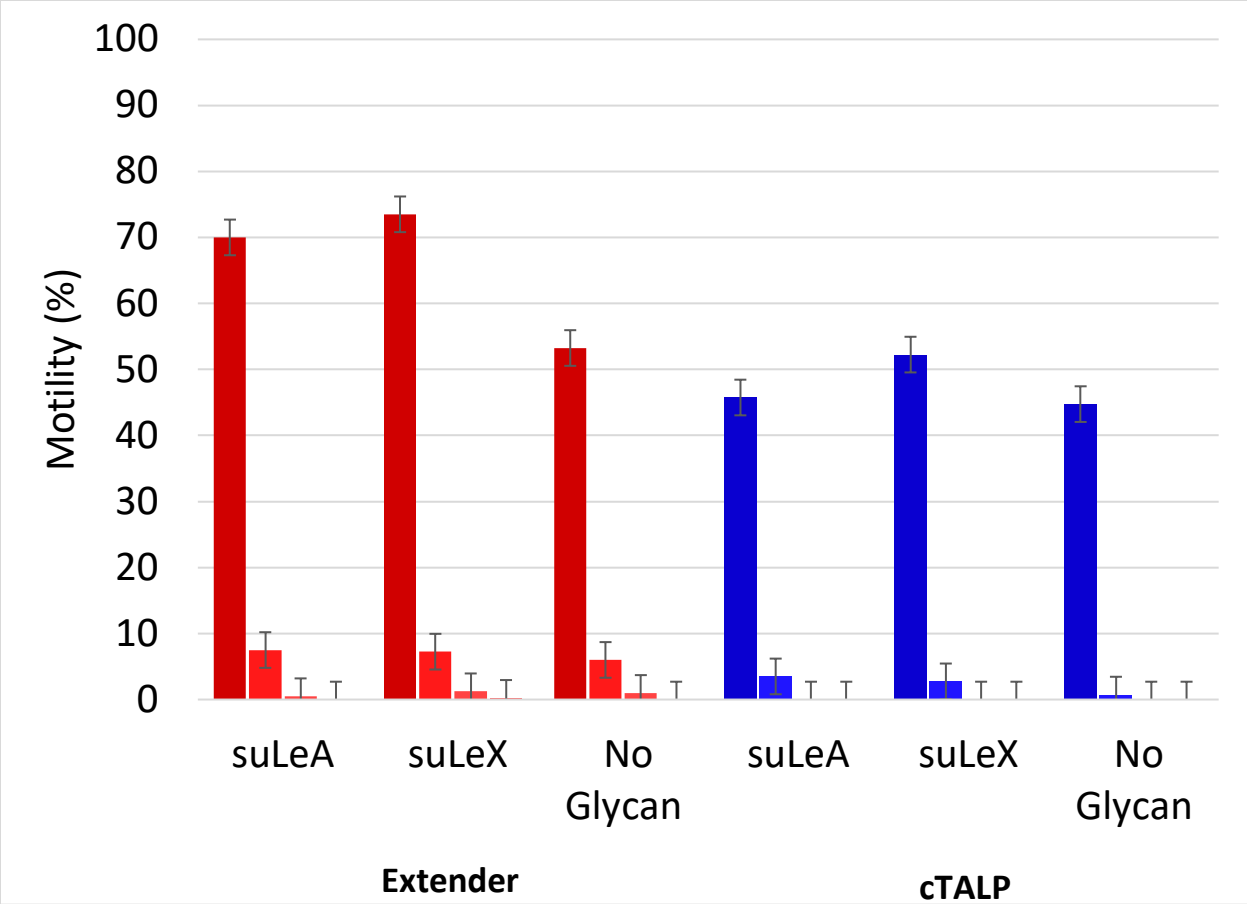

Figure C.8: Motility of Bull Sperm at 37°C over a 120 hr time period. Graph representing the average viability of pooled bull semen with 3 soluble glycan treatments and 2 media treatments at 4 time points at 37°C. Treatments in red were in FSRD4+ extender, while treatments in blue were in dmTALPC. From left to right, the treatments are $suLe^A$ in FSRD4+, $suLe^X$ in FSRD4+, no glycan in FSRD4+, $suLe^A$ in dmTALPC, $suLe^X$ in dmTALPC, and no glycan in dmTALPC. The different shades represent the 4 time points: 1 hr, 24 hr, 48 hr, and 72 hr. The motility for all treatments at 96 hr and 120 hr was 0%; therefore, both time points were removed from this graph.

APPENDIX D – PROTOCOL FOR DETERMINING VIABILITY AND MOTILITY AFTER SPERM INCUBATION

Plate Preparation

1. Label 4 96-well plates; each plate represents a specific temperature (5°C, 15°C, 22°C, and 37°C).
2. Label the columns of each plate with each media/glycan combination (soluble $suLe^X$/extender, soluble $suLe^A$/extender, no glycans/extender, soluble $suLe^X$/dmTALPC, soluble $suLe^A$/dmTALPC, no glycans/dmTALPC).
3. Label the rows of each plate with hour counts from 1-120 hours every 24 hr, i.e., 1, 24, 48, 72, 96, and 120 hr.
4. In each of the wells labeled with a specific glycan, pipette in 3 μL of the respective soluble glycans, if applicable.

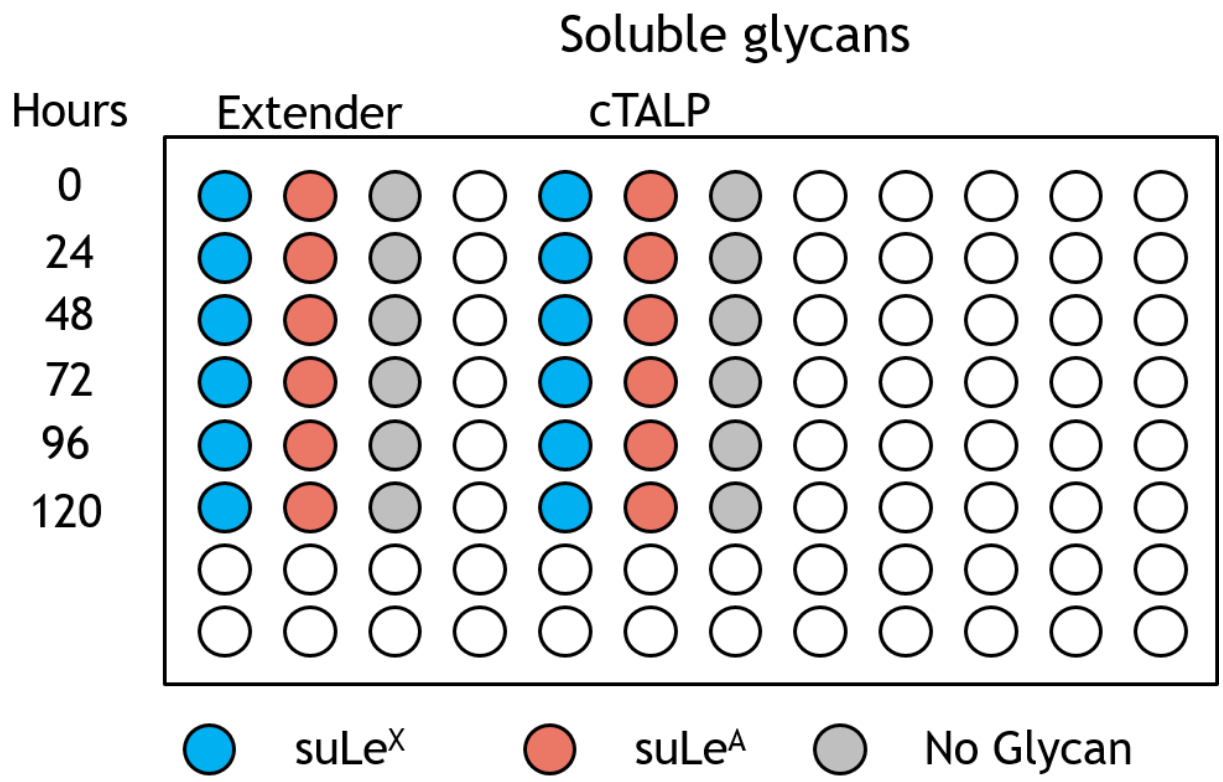

Figure D.1: An aerial view of a 96-well plate with the soluble glycans.

Sperm Preparation

1. Prepare 5 15-mL conical tubes for each of the following contents: bull semen, Percoll wash, dmTALPC for the trials, dmTALPC for the sperm washes, and FSRD4+ extender.
2. Warm 10 mL of dmTALPC for the trials, 15 mL of dmTALPC for the washing, 10 mL of FSRD4+ extender, 4 mL of bull semen, and 10 mL of Percoll wash at 37°C for 15 minutes.
3. Pipette 3 mL of the bull semen onto the top of the Percoll wash; do not mix the Percoll and semen.
4. Centrifuge the tube at 796xg for 12 min.
5. Discard the supernatant and resuspend the pellet with 5 mL of warmed dmTALPC.

6. Centrifuge the tube at 195xg for 5 min.
7. Discard the supernatant and resuspend the pellet with 5 mL of warmed dmTALPC.
8. Centrifuge the tube at 195xg for 5 min for a second time.
9. Discard the supernatant and resuspend the pellet with 5 mL of warmed dmTALPC.
10. Using the hemocytometer, determine the sperm count and dilute the washed sperm to a concentration of 2.0 x 10^6 sperm/mL in both the dmTALPC and FSRD4+ extender conical tubes.
11. Add 57 μL of the diluted sperm to each labeled wells for all four of the 96-well plates.
    a. The glycan concentration with the sperm will be at 50 μg/mL.
    b. The total well volume will be at 60 μL for every well with glycan treatments.
12. Place one 96-well plate in each of their respective temperatures (5°C, 15°C, 22°C, and 37°C) in the dark to prevent light from inactivating the bactericide/fungicide.
13. After the incubation period is over, prepare the sperm for either viability counts or motility counts.

Viability Preparation and Counting

1. 15 min prior to the end of the incubation period, 30 μL of each treatment from every plate is transferred to 1.5 mL black microcentrifuge tubes.
2. Prepare the two fluorescent stains SYBR14 and propidium iodide (PI) at concentrations of 20 μM and 800 μM respectively.
3. Add 1μL of SYBR14 to every tube and incubate the tubes at 37°C for 12 min.
4. Take out the tubes and pipette 1 μL of PI to every tube and incubate the tubes at 37°C for 3 min.

5. Prepare individual slides in the dark for every treatment by placing 20 µL on the slide and covering with a coverslip.
6. Through the eyepiece of the Zeiss Axioskop (Zeiss Microscopy, LLC, Thornwood, NY) at 200X magnification, count 100 total sperm, either fluorescing green to indicate it is alive or fluorescing red to indicate it is dead.

Motility Preparation and Counting

1. After incubation, pipette 25 µL of every treatment group into individual 1.5 mL microcentrifuge tubes.
2. Place the tubes into an incubator at 37°C for 15 minutes.
3. Pipette out 25 µL onto a warmed slide and cover with a coverslip.
4. Place the slide on a microscope stage at 100X magnification and quickly count 100 sperm to record the percentage of motile cells for each treatment group.

APPENDIX E – COOMASSIE BLUE STAINING FOR ACROSOMAL STATUS

1. Place 10 µL of sperm suspension in a microcentrifuge tube and add 25 µL of 4% paraformaldehyde.
2. Incubate for 10 min at room temperature to fix the cells.
3. Centrifuge at 2000xg for 1 min.
4. Discard the supernatant and resuspend the pellet in 50 µL of 0.1M ammonium acetate.
5. Centrifuge at 2000xg for 1 min.
6. Discard supernatant and resuspend pellet in 25 µL of 0.1M ammonium acetate.
7. Place 10 µL of sperm suspension on each slide and allow to air dry on a slide warmer.
8. Dip the dried slides in Coomassie Blue G-250 Solution (0.22% Coomassie Blue G-250, 50% methanol, 10% glacial acetic acid, and 40% water) in a Coplin jar for 2-4 min and rinse the slides with deionized water to remove the stain. Allow the slides to air dry.
9. Add a drop of 1X PBS and top the slide with a coverslip.
10. Take pictures of ~300 sperm and record the acrosomal status.

APPENDIX F – FSRD4+ EXTENDER PREPARATION PROTOCOL

1. Prior to preparing the FSRD4+ extender, all instruments that can autoclaved are autoclaved at 121°C for 1 hr.
    a. The instruments used were 100 mL Erlenmeyer flask, spatula, 100 mL beaker, 100 mL graduated cylinder, glass bottle with cap for storage, one magnetic stir bar, and pipette tips.
2. Any other instruments were wiped down with 70% ethanol before being used under a biological safety cabinet.
3. The extender is taken from the source and measured out in a 100 mL graduated cylinder as 95% of the total volume needed for extender preparation.
    a. Note: Roughly 15% of the extender will be lost due to transferring solutions and centrifugation.
    b. For example, add 5.75 mL of egg yolk to 109.25 mL of extender for a total volume of 115mL before centrifugation. After the extender is fully prepared, the end total volume will be roughly 100 mL.
4. To extract the egg yolk, carefully crack the egg and remove as much of the egg white as possible.
5. Place the egg yolk on disc paper and carefully roll the yolk to expose a surface with no egg white on the vitelline membrane.
6. Using a disposable pipette, suction out 5% of egg yolk and transfer to the 100 mL graduated cylinder.
7. Pour the contents from the 100 mL graduated cylinder into the 100 mL beaker with the magnetic stir bar in the beaker.

8. Turn on the stir plate and allow the extender to mix for 15 min at room temperature.
9. Bubble N_2 gas through the extender for 30 min to allow the dissolved oxygen to be replaced with nitrogen.
10. Either store the extender overnight at 4°C or store at 4°C for 4 hr to allow sedimentation.
11. Pipette equal volumes of extender into polypropylene tubes for ultracentrifugation.
12. Centrifuge the extender for 1 hr at 4°C at 9200xg.
13. Carefully remove the tubes from the rotor to avoid disturbing the sediment.
14. Combine all of the extender aliquots into a single conical tube, if possible.
15. Check the pH of the extender and be in the range of 6.8-7.1.
16. Top off the conical tube with N_2 gas before storing the extender at 4°C.
    a. The shelf life of the freshly prepared extender is 5 days.
    b. The extender needs to be fully prepared at least 24 hr before using.